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Single-Dose Pharmacokinetics of Clobenzorex & Its Metabolites

u/TheMaengDaDuke

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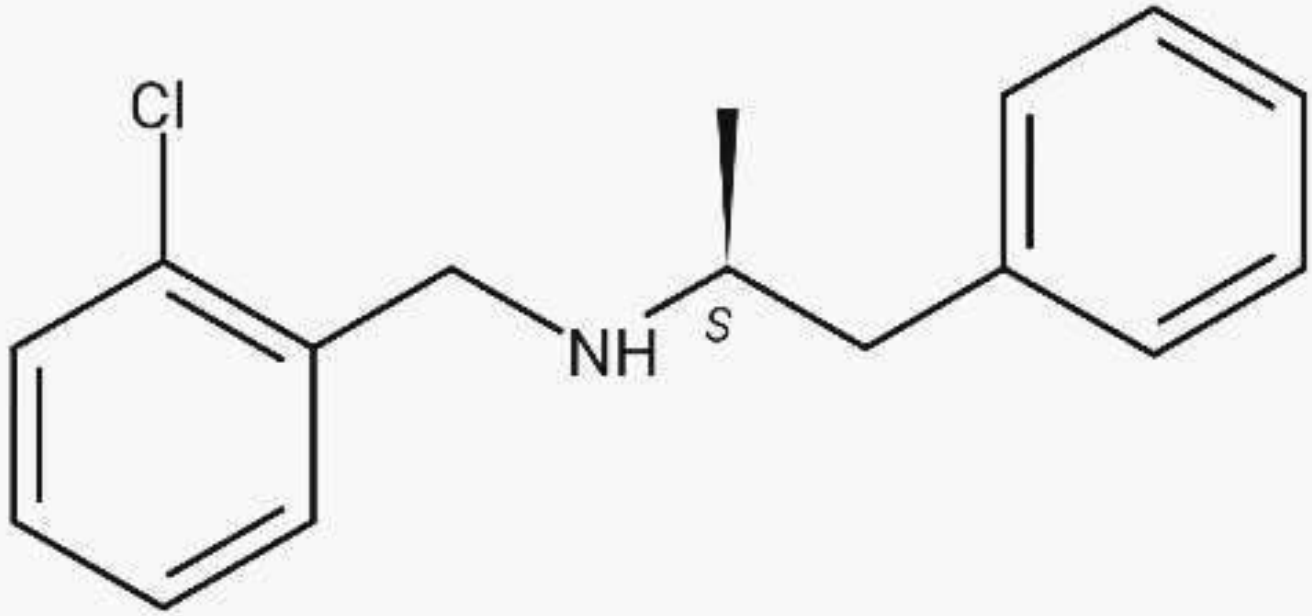

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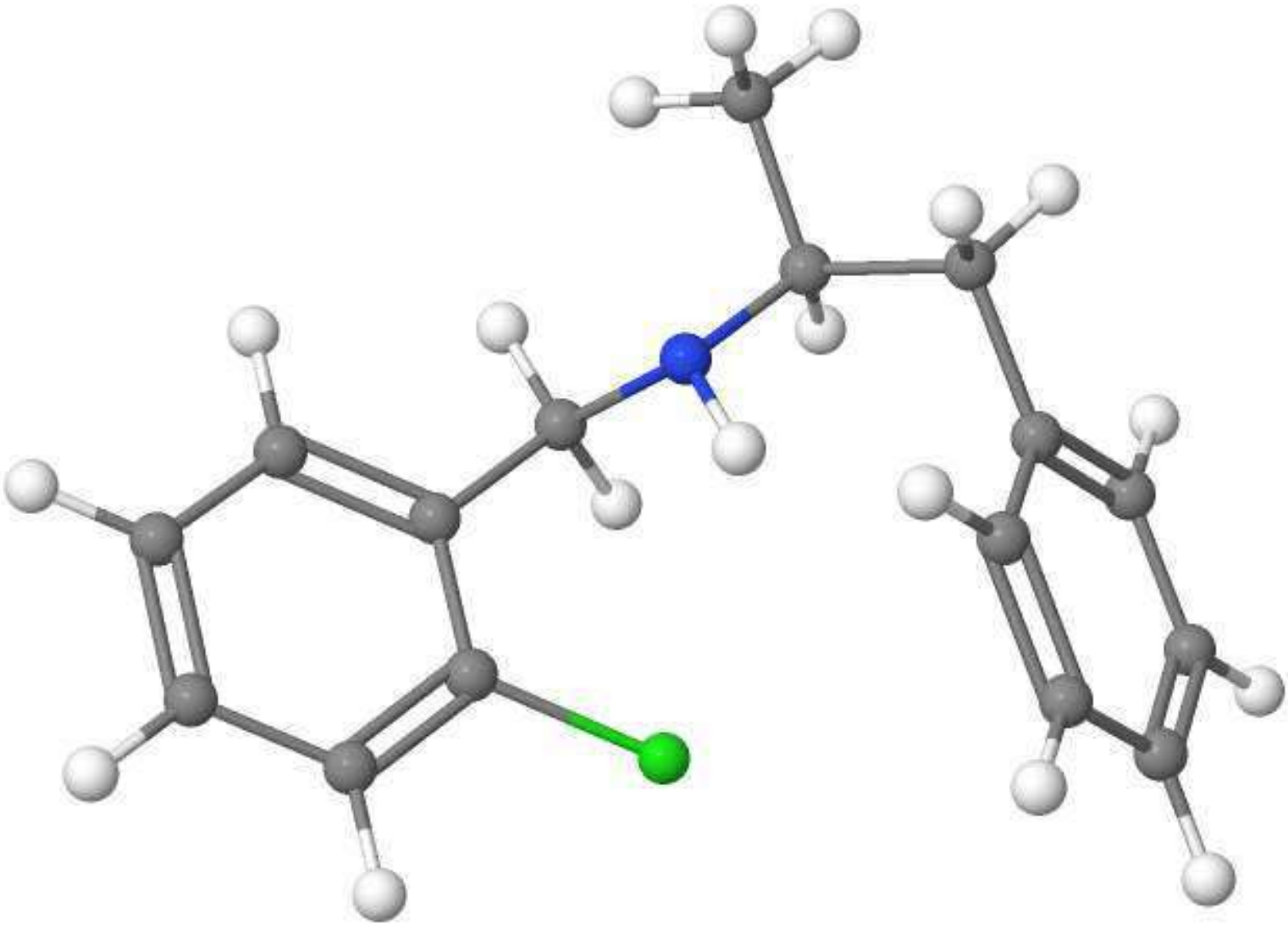

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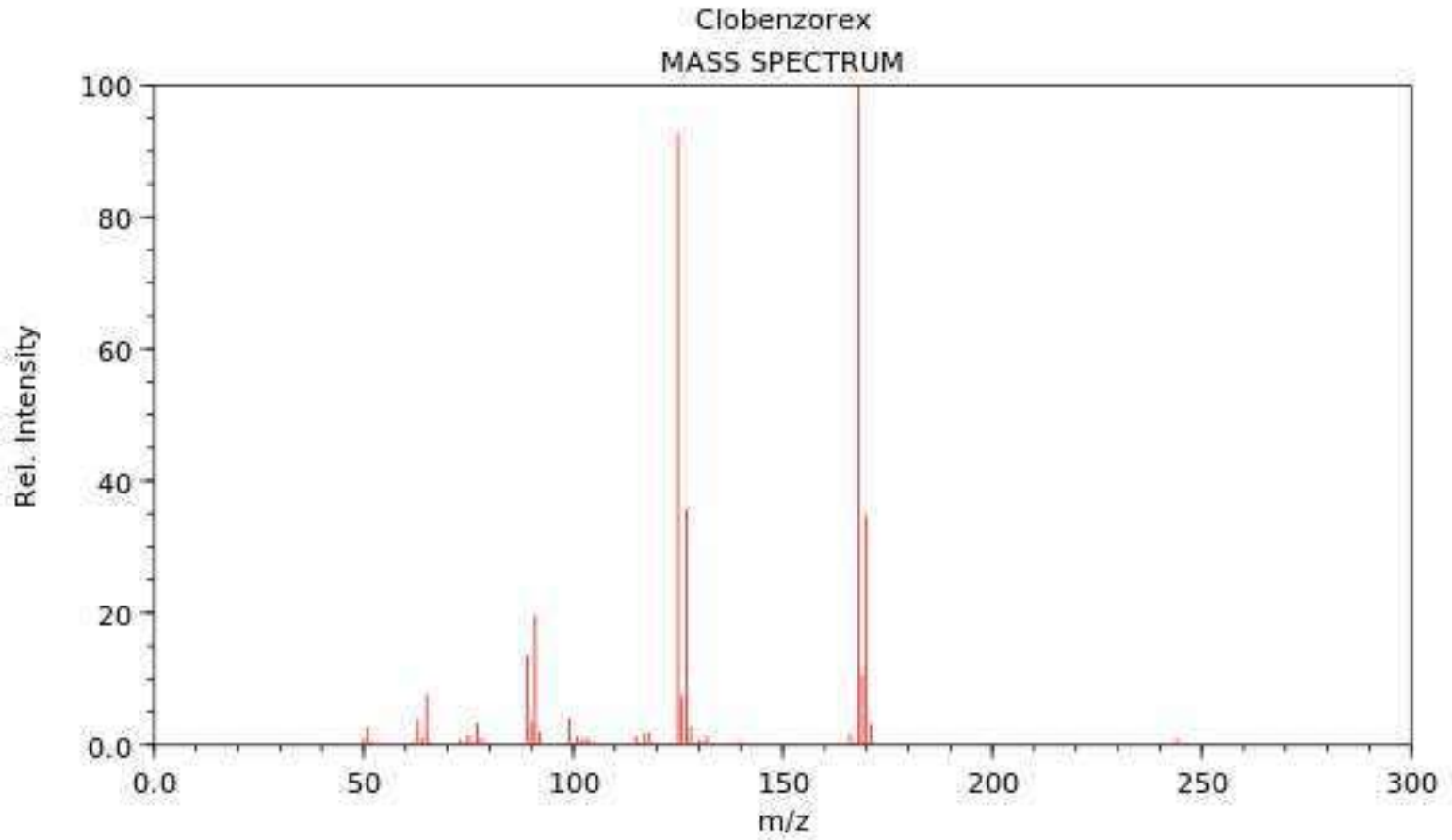

NIST Chemistry WebBook (https://webbook.nist.gov/chemistry)

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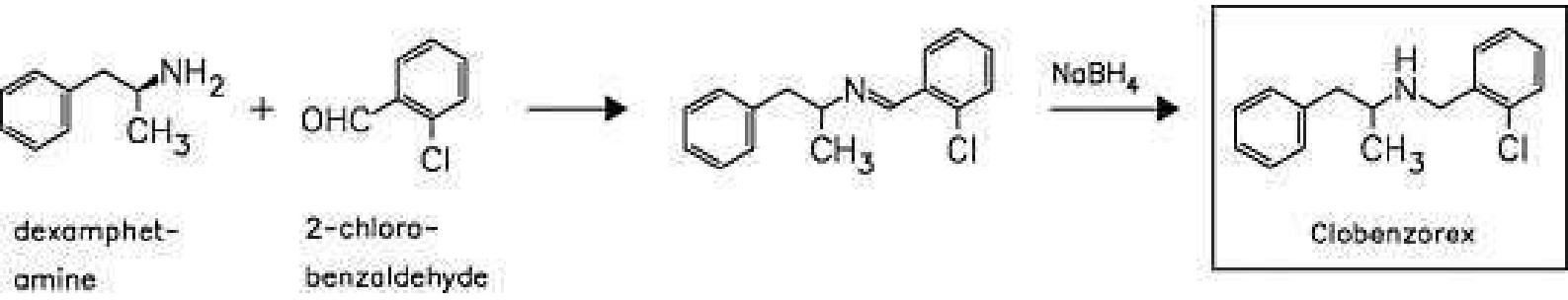

dexamphet-amine

2-chloro-benzaldehyde

Clobenzorex

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FÓRMULA: Cada cápsula contiene:
Clorhidrato de Clobenzorex ..... 30 mg
Excipiente cbp ..... 1 cápsula
DOSIS: La que el médico señale
VÍA DE ADMINISTRACIÓN: Oral
Su venta requiere receta médica que podrá surtirse en tres ocasiones con vigencia de seis meses
Su uso prolongado aún a dosis terapéuticas puede causar dependencia
Este medicamento contiene Azul No. 1, que puede producir reacciones de hipersensibilidad
Reporte las sospechas de reacción adversa al correo: farmacovigilancia@cofepris.gob.mx y farmacovigilancia@ifa.com.mx

No se deje al alcance de los niños
No se administre durante el embarazo y la lactancia
Consérvese a no más de 30°C, en lugar seco y conserve la caja bien cerrada
Léase instructivo anexo
Reg. No. 479M99 SSA III

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Asenlix®

Fórmula:
Cada cápsula contiene:

Clorhidrato de Clobenzorex	30 mg
Excipiente cbp	1 cápsula

Dosis: La que el médico señale.
Léase instructivo anexo. Su venta requiere receta médica, que podrá surtirse en tres ocasiones con vigencia de seis meses. Su uso prolongado, aún a dosis terapéuticas, puede causar dependencia. No se deje al alcance de los niños. No se use en el embarazo, lactancia, ni en menores de 12 años. Consérvese la caja bien cerrada a no más de 30 °C y en lugar seco. Reporte las sospechas de reacción adversa a los correos: farmacovigilancia@cofepris.gob.mx y ucfarmacovigilancia@hormona.com.mx

Reg. No. 82946 SSA III

®Marca Registrada

Hecho en México por: **Grimann, S.A. de C.V.**
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HORMONA LABORATORIOS

Safety Data Sheet - Version 5.0

Preparation Date 9/9/2019

Latest Revision Date (If Revised)

1. IDENTIFICATION OF THE SUBSTANCE/MIXTURE AND OF THE COMPANY/UNDERTAKING

1.1 Product Identifier

Chemical Name (S)-(+)-Clobenzorex Hydrochloride

Catalogue # C582990

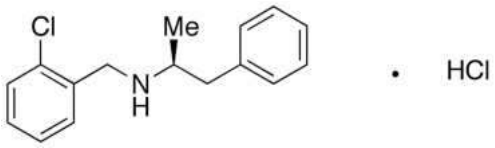
1.2 Relevant Identified Uses of the Substance or Mixture and Uses Advised Against

Product Uses To be used only for scientific research and development. Not for use in humans or animals.

1.3 Details of the Supplier of the Safety Data Sheet

Company Toronto Research Chemicals
2 Brisbane Road
Toronto, ON M3J 2J8
CANADA

Telephone +14166659696
FAX +14166654439
Email orders.trc@lgcgroup.com



1.4 Emergency Telephone Number

Emergency# +1(416) 665-9696 between 0800-1700 (GMT-5)

2. HAZARDS IDENTIFICATION

2.1/2.2 Classification of the Substance or Mixture and Label Elements

GHS Hazards Classification (According to EU Regulation 1272/2008 and US OSHA 1910.1200)

Acute Toxicity, Oral (Category 4)

GHS Hazards Identification (According to EU Regulation 1272/2008 and US OSHA 1910.1200)

Signal Word Warning

GHS Hazard Statements

H302 Harmful if swallowed.

GHS Precautionary Statements

P264 Wash hands thoroughly after handling.

P301/P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.

2.3 Unclassified Hazards/Hazards Not Otherwise Classified

No data available.

3. COMPOSITION/INFORMATION ON INGREDIENTS

3.1 Substances

Molecular Formula: C□□H□□Cl□N **Molecular Weight:** 296.23

CAS Registry #: 5843-53-8 **EC#:** 227-434-5

Synonyms

(αS)-N-[(2-Chlorophenyl)methyl]-α-methylbenzeneethanamine Hydrochloride; (+)-N-(o-Chlorobenzyl)-α-methylphenethylamine Hydrochloride; Asenlix; Ba 7205; Clobenzorex Hydrochloride; Dinintel; Rexigen; SD 271-12;

3.2 Mixtures

Not a mixture.

4. FIRST AID MEASURES

4.1 Description of First Aid Measures

General Advice
If medical attention is required, show this safety data sheet to the doctor.

If Inhaled
If inhaled, move person to fresh air. If not breathing, give artificial respiration and consult a physician.

In Case of Skin Contact
Wash affected area with soap and water. Consult a physician if any exposure symptoms are observed.

In Case of Eye Contact
Immediately rinse eyes with plenty of water for at least 15 minutes. Consult a physician.

If Swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Do NOT induce vomiting unless advised to do so by a physician or Poison Control Center. Seek medical attention.

4.2 Most Important Symptoms and Effects, Both Acute and Delayed

The most important known symptoms and effects are described in the labeling (see section 2.2) and/or section 11.

4.3 Indication of any Immediate Medical Attention and Special Treatment Needed

No data available.

5. FIREFIGHTING MEASURES

5.1 Extinguishing Media

Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

5.2 Special Hazards Arising from the Substance or Mixture

Carbon oxides, Nitrogen oxides, Hydrogen chloride

5.3 Advice for Firefighters

Wear self contained breathing apparatus for fire fighting if necessary. Use personal protection equipment.

5.4 Further Information

No data available.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Wear respiratory protection. Avoid dust formation. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust. Avoid contact with skin, eyes or clothing.

Environmental precautions
Prevent further leakage or spillage if safe to do so. Do not let product enter drains.

Method and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

7.1 Precautions for safe handling

Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed.

7.2 Conditions for safe storage

Keep container tightly closed in a dry and well-ventilated place.
Keep in a dry place.

Storage conditions: -20°C

7.3 Specific End Uses

For scientific research and development only. Not for use in humans or animals.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

8.1 Control Parameters

Contains no components with established occupational exposure limits.

8.2 Exposure Controls

Appropriate Engineering Controls

A local exhaust hood or other appropriate form of local exhaust ventilation should be used to avoid exposure.

Personal Protective Equipment

All recommendations below are advisory in nature and a risk assessment should be performed by the employer/end user prior to use of this product. The type of protective equipment must be selected based on the amount and concentration of the dangerous material being used in the workplace.

Eye/Face Protection

Safety goggles or face shield. All equipment should have been tested and approved under appropriate standards, such as NIOSH (US), CSA (Canada), or EN 166 (EU).

Skin Protection

Gloves should be used when handling this material. Gloves are to be inspected prior to use. Contaminated gloves are to be removed using proper glove removal technique so that the outer surface of the glove does not contact bare skin. Dispose of contaminated gloves after use in compliance with good laboratory practices and local requirements.

Gloves used for incidental exposures (splash protection) should be designated as “chemical resistant” by EU standard EN 374 with the resistance codes corresponding to the anticipated use of the material. Unrated gloves are not recommended.
Suggested gloves: AnsellPro Sol-Vex nitrile gloves style 37-175, 15 mil thickness.
Penetration time has not been determined.

Gloves used for prolonged direct exposure (immersion) should be designated “chemical resistant” as per EN 734 with the resistance codes corresponding to the anticipated use of the material.
Suggested gloves: AnsellPro Viton/Butyl gloves style 38-612, 4/8 mil thickness.
Penetration time has not been determined.

These recommendations may not apply if the material is mixed with any other chemical, or dissolved into a solution. A risk assessment must be performed to ensure the gloves will still offer acceptable protection.

Body Protection

Fire resistant (Nomex) lab coat or coveralls.

Respiratory Protection

Recommended respirators are NIOSH-approved N100 or CEN-approved FFP3 particulate respirators. These are to be only used as a backup to local exhaust ventilation or other engineering controls. If the respirator is the only means of protection, a full-face supplied air respirator must be used.

9. PHYSICAL AND CHEMICAL PROPERTIES

9.1 Information on Basic Physical and Chemical Properties

A) Appearance
White to Off-White Solid

B) Odour
No data available

C) Odour Threshold
No data available

D) pH
No data available

E) Melting Point/Freezing Point
181 - 183°C

F) Initial Boiling Point/Boiling Range
No data available

G) Flash point
No data available

H) Evaporation Rate
No data available

I) Flammability (Solid/Gas)
No data available

J) Upper/Lower Flammability/Explosive Limits
No data available

K) Vapour Pressure
No data available

L) Vapour Density
No data available

M) Relative Density
No data available

N) Solubility
Methanol (Slightly), Water (Slightly, Heated)

O) Partition Coefficient: n-octanol/water
No data available

P) Auto-Ignition Temperature
No data available

Q) Decomposition Temperature
No data available

R) Viscosity
No data available

S) Explosive Properties
No data available

T) Oxidizing Properties
No data available

9.2 Other Information

no data available

10. STABILITY AND REACTIVITY

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10.1 Reactivity
No data available.

10.2 Chemical Stability

Stable under recommended storage conditions.

10.3 Possibility of Hazardous Reactions

No data available.

10.4 Conditions to Avoid

No data available.

10.5 Incompatible Materials

Strong oxidizing agents.

10.6 Hazardous Decomposition Products

In the event of fire: See section 5. **Other decomposition products:** No data available.

11. TOXICOLOGICAL INFORMATION

11.1 Information on Toxicological Effects

A) Acute Toxicity

Oral LD50: No data available

Dermal LD50: No data available

Inhalation LC50: No data available

B) Skin Corrosion/Irritation

No data available

C) Serious Eye Damage/Irritation

No data available

D) Respiratory or Skin Sensitization

No data available

E) Germ Cell Mutagenicity

No data available

F) Carcinogenicity

No data available

G) Reproductive Toxicity/Teratogenicity

No data available

H) Single Target Organ Toxicity - Single Exposure

No data available

I) Single Target Organ Toxicity - Repeated Exposure

No data available

J) Aspiration Hazard

No data available

K) Potential Health Effects and Routes of Exposure

Inhalation

May be harmful if inhaled. May cause respiratory tract irritation.

Ingestion

May be harmful if swallowed.

Skin

May be harmful if absorbed through skin. May cause skin irritation.

Eyes

May cause eye irritation.

L) Signs and Symptoms of Exposure

The most important known symptoms and effects are described in the labeling (see section 2.2) and/or section 11.

To the best of our knowledge, the chemical, physical, and toxicological properties of this material have not been thoroughly investigated.

M) Additional Information

RTECS: SH0683700

12. ECOLOGICAL INFORMATION

12.1 Toxicity

No data available.

12.2 Persistance and Degradability

No data available.

12.3 Bioaccumulative Potential

No data available.

12.4 Mobility in Soil

No data available.

12.5 Results of PBT and vPvB Assessment

No data available.

12.6 Other Adverse Effects

No data available.

13. DISPOSAL CONSIDERATIONS

13.1 Waste Treatment Methods

A) Product

Product may be burned in an incinerator equipped with afterburner and scrubber. Excess and expired materials are to be offered to a licensed hazardous material disposal company. Ensure that all Federal and Local regulations regarding the disposal and destruction of this material are followed.

B) Contaminated Packaging

Dispose of as above.

C) Other Considerations

Product is not to be disposed of in sanitary sewers, storm sewers, or landfills.

14. TRANSPORT INFORMATION

14.1 UN Number

DOT (US): N/A IATA: N/A IMDG: N/A ADR/RID: N/A

14.2 UN Proper Shipping Name

DOT (US)/IATA:

Not dangerous goods

IMDG/ARD/RID:

Not dangerous goods

14.3 Transport Hazard Class(es)

DOT (US): N/A IATA: N/A IMDG: N/A ADR/RID: N/A

14.4 Packing Group

DOT (US): N/A IATA: N/A IMDG: N/A ADR/RID: N/A

14.5 Environmental Hazards

DOT (US): None IATA: None IMDG: None ADR/RID: None

14.6 Special Precautions for User

None

15. REGULATORY INFORMATION

This safety data sheet complies with the requirements of WHMIS (Canada), OSHA 1910.1200 (US), and EU Regulation EC No. 1907/2006 (European Union).

15.1 Safety, Health and Environmental Regulations/Legislation Specific for the Substance or Mixture

A) Canada

DSL/NDSL Status: This product is not listed on the Canadian DSL/NDSL.

B) United States

TSCA Status: This product is not listed on the US EPA TSCA.

C) European Union

ECHA Status: This product is not registered with the EU ECHA.

15.2 Chemical Safety Assessment

No data available

16. OTHER INFORMATION

16.1 Revision History

Original Publication Date: 9/9/2019

16.2 List of Abbreviations

LD50	Median lethal dose of a substance required to kill 50% of a test population.
LC50	Medial lethal concentration of a substance required to kill 50% of a test population.
LDLo	Lowest known lethal dose
TDLo	Lowest known toxic dose

This Safety Data Sheet contains 16 sections. All 16 sections must be present for this document to be valid.

IARC International Agency for Research on Cancer
NTP National Toxicology Program
RTECS Registry of Toxic Effects of Chemical Substances

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16.3 Further Information

Copyright 2015. Toronto Research Chemicals Inc. Copies may be made for internal use only. The above information is believed to be correct to the best of our knowledge, but is to be only used as a guide. To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated. Please take all due care when handling this product.

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Australian Government
Department of Industry, Science and Resources

National Measurement Institute

NATA
WORLD RECOGNISED ACCREDITATION

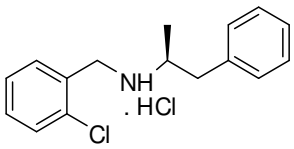
CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

NMIA D692b: Clobenzorex hydrochloride

Report ID: D692b.2019.03

Chemical Formula: $C_{16}H_{18}NCl.HCl$

Molecular Weight: 296.2 g/mol (HCl), 259.8 g/mol (base)



Certified value

Batch No.	CAS No.	Purity (mass fraction)
09-D-09	5843-53-8 (HCl) 13364-32-4 (base)	99.6 ± 0.5%

The uncertainty has been calculated according to ISO Guide 35 and is stated at the 95% confidence limit (k = 2).

Synonyms: (+)-*N*-[(2-Chlorophenyl)methyl]-α-methylbenzeneethanamine hydrochloride
(+)-*N*-[(*o*-Chlorobenzyl)-α-methylphenethylamine hydrochloride
d-*N*-(1-Phenyl-2-propyl)-2-chlorobenzylamine
(*S*)-*N*-[(2-Chlorophenyl)methyl]-α-methylbenzeneethanamine hydrochloride

Expiration of certification: The property values are valid till 5 November 2024, i.e. five years from the date of re-certification provided the **unopened** material is handled and stored in accordance with the recommendations below. The material as issued in the unopened container and stored as recommended below should be suitable for use beyond this date, subject to confirmation of batch stability from the issuing body. The expiry date/shelf life does not apply to sample bottles that have been opened. In such cases it is recommended that the end-user conduct their own in-house stability trials.

Description: White crystalline flakes prepared by synthesis, and certified for identity and purity by NMIA. Packaged in amber glass bottles with a septum and crimped aluminium cap or screw top cap.

Intended use: This certified reference material is suitable for use as a primary calibrator.

Instructions for use: Equilibrate the bottled material to room temperature before opening.

Recommended storage: When not in use this material should be stored at or below 25 ºC in a closed container in a dry, dark area.

Metrological traceability: The certified purity value is traceable to the SI unit for mass (kg) through Australian national standards via balance calibration. In the mass balance approach all impurities are quantified as a mass fraction and subtracted from 100%. Quantitative NMR provides an independent direct measure of the mass fraction of the analyte of interest, calibrated with an internal standard certified for purity (mass fraction).

Stability: This material has demonstrated stability over a minimum period of three years. The measurement uncertainty at the 95% confidence interval includes a stability component which has been estimated from annual stability trials. The long-term stability of the compound in solution has not been examined.

Homogeneity assessment: The homogeneity of the material was assessed using purity assay by GC-FID on seven randomly selected 1-2 mg sub samples of the material. The material was judged to be sufficiently homogeneous at this level of sampling as the variation in analysis results between samples was not significantly different at a 95% confidence level from that observed on repeat analysis of the same sample.

Safety: Treat as a hazardous substance. Use appropriate work practices when handling to avoid skin or eye contact, ingestion or inhalation of dust. Refer to the provided safety data sheet.

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S. R. Davies

Dr Stephen R. Davies,
Team Leader,
Chemical Reference Materials, NMI.
14 September 2022

This report supersedes any issued prior to 14 September 2022.

NATA Accreditation No. 198 / Corporate Site No. 14214.

Legal notice: Terms and Conditions associated with the provision of this reference material can be found on the NMIA website.

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Characterisation Report:

The identity was confirmed by a range of spectroscopic techniques, NMR, IR and MS. The certified purity value was obtained by mass balance from a combination of traditional analytical techniques, including GC-FID, thermogravimetric analysis, Karl Fischer analysis and ^{1}H NMR spectroscopy. The purity value is calculated as per Equation 1.

Purity = (100 % - I_{ORG}) x (100 % - I_{VOL} – I_{NVR}) Equation 1

I_{ORG} = Organic impurities of related structure, I_{VOL} = volatile impurities, I_{NVR} = non-volatile residue.

Supporting evidence is provided by qualitative elemental microanalysis.

GC-FID:	Instrument:	Agilent 6890N
	Column:	HP-1, 30 m × 0.32 mm I.D. × 0.25 μm
	Program:	100 °C (1 min), 10 °C/min to 240 °C, 30 °C/min to 300 °C (3 min)
	Injector:	250 °C
	Detector Temp:	320 °C
	Carrier:	Helium
	Split ratio:	20/1
	Relative mass fraction of the main component:	
	Initial analysis:	Mean = 99.2%, s = 0.05% (7 sub samples in duplicate, April 2009)
	Re-analysis:	Mean = 99.5%, s = 0.04% (4 sub samples in duplicate, February 2012)
	Re-analysis:	Mean = 99.6%, s = 0.01% (5 sub samples in duplicate, December 2014)
	Re-analysis:	Mean = 99.7%, s = 0.01% (5 sub samples in duplicate, November 2019)
GC-FID:	Instrument:	Agilent 6890N
	Column:	HP-1, 30 m × 0.32 mm I.D. × 0.25 μm
	Program:	100 °C (1 min), 10 °C/min to 160 °C, 2 °C/min to 180 °C, 5 °C/min to 240 °C, 30 °C/min to 300 °C (3 min)
	Injector:	250 °C
	Detector Temp:	320 °C
	Carrier:	Helium
	Split ratio:	20/1
	Relative mass fraction of the main component:	
	Initial analysis:	Mean = 99.3%, s = 0.02% (5 sub samples in duplicate, April 2010)
Thermogravimetric analysis:		Volatile content not determined due to inherent volatility of the material
Karl Fischer analysis:		Moisture content ≤ 0.2% mass fraction (April 2009, April 2010, May 2011, January 2015 and September 2019)

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Spectroscopic and other characterisation data

GC-MS:	Instrument:	Agilent 6890
	Column:	VF-1ms, 14.9 m × 0.25 mm I.D. × 0.25 μm
	Program:	80 °C (1 min), 10 °C/min to 250 °C, 40 °C/min to 300 °C (2 min)
	Injector:	250 °C
	Transfer line temp:	300 °C
	Carrier:	Helium, 1.0 mL/min
	Split ratio:	20/1
	The retention time of the parent compound is reported along with the major peaks in the mass spectrum. The latter are reported as mass/charge ratios and (in brackets) as a percentage relative to the base peak.	
	11.9 min:	170 (28), 168 (88), 127 (33), 125 (100), 91 (22), 89 (13) *m/z*
ESI-MS:	Instrument:	Micromass Quatro Micro
	Operation:	Positive ion mode, direct infusion at 5 μL/min
	Ionisation:	ESI spray voltage at 3.5 kV positive ion
	EM voltage:	650 V
	Cone voltage:	20 V
	Peak:	260 ($M+H^+$, ^{35}Cl, 100) *m/z*
TLC:	Conditions:	Kieselgel 60F_{254}. Hexane/diethylamine (20/1) Single spot observed, R_f = 0.5. Visualisation with UV at 254 nm
IR:	Instrument:	Biorad FTS300MX FT-IR
	Range:	4000-400cm^{-1}, KBr powder
	Peaks:	2932, 2788, 2547, 2402, 1574, 1447, 1387, 1088, 1055, 985, 838, 757, 743, 698, 465 cm^{-1}
^{1}H NMR:	Instrument:	Bruker Avance III
	Field strength:	500 MHz
	Solvent:	MeOH-*d*$_4$ (3.31 ppm)
	Spectral data:	δ 1.34 (3H, d, *J* = 6.5 Hz), 2.81 (1H, dd, *J* = 10.6, 13.2 Hz), 3.37 (1H, dd, *J* = 4.4, 13.2 Hz), 3.67 (1H, m), 4.46 (1H, d, *J* = 13.4 Hz), 4.47 (1H, d, *J* = 13.4 Hz), 7.27-7.31 (3H, m), 7.34-7.39 (2H, m), 7.43-7.51 (2H, m), 7.57 (1H, dd, *J* = 1.5, 7.8 Hz), 7.71 (1H, d, *J* = 7.5 Hz) ppm
		Isopropanol was quantified at ≤ 0.2% mass fraction.
^{13}C NMR:	Instrument:	Bruker Gyro 300
	Field strength:	75 MHz
	Solvent:	MeOH-*d*$_4$ (49.00 ppm)
	Spectral data:	δ 16.0, 40.1, 47.1, 57.8, 128.4, 129.1, 130.0, 130.4, 130.8, 131.2, 132.6, 133.5, 135.9, 137.3 ppm
Melting point:		181-183 °C
Microanalysis:	Found:	C = 65.2%; H = 6.8%; N = 4.7% (April 2009)
	Calculated:	C = 64.9%; H = 6.5%; N = 4.7% (Calculated for $C_{16}H_{19}Cl_2N$)

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Clobenzorex, AC

Inchi: InChI=1S/C18H20ClNO/c1-14(12-16-8-4-3-5-9-16)20(15(2)21)13-17-10-6-7-11-18(17)19/
InchiKey: KRTYUJXALHAFCT-UHFFFAOYSA-N
Formula: C18H20ClNO
SMILES: CC(=O)N(Cc1ccccc1Cl)C(C)Cc1ccccc1
Mol. weight [g/mol]: 301.81

Physical Properties

Property code	Value	Unit	Source
gf	283.36	kJ/mol	Joback Method
hf	-19.33	kJ/mol	Joback Method
hfus	35.36	kJ/mol	Joback Method
hvap	73.66	kJ/mol	Joback Method
log10ws	-5.20		Crippen Method
logp	4.320		Crippen Method
mcvol	240.750	ml/mol	McGowan Method
pc	1945.79	kPa	Joback Method
tb	772.88	K	Joback Method
tc	1004.68	K	Joback Method
tf	455.30	K	Joback Method
vc	0.894	m3/kmol	Joback Method

Temperature Dependent Properties

Property code	Value	Unit	Temperature [K]	Source
cpg	666.14	J/mol×K	772.88	Joback Method
cpg	682.11	J/mol×K	811.51	Joback Method
cpg	696.83	J/mol×K	850.15	Joback Method
cpg	710.38	J/mol×K	888.78	Joback Method
cpg	722.86	J/mol×K	927.41	Joback Method
cpg	734.36	J/mol×K	966.04	Joback Method
cpg	744.97	J/mol×K	1004.68	Joback Method

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Sources

Crippen Method:	http://pubs.acs.org/doi/abs/10.1021/ci990307l
Crippen Method:	https://www.chemeo.com/doc/models/crippen_log10ws
Joback Method:	https://en.wikipedia.org/wiki/Joback_method
McGowan Method:	http://link.springer.com/article/10.1007/BF02311772
NIST Webbook:	http://webbook.nist.gov/cgi/cbook.cgi?ID=R269413&Units=SI

Legend

cpg:	Ideal gas heat capacity
gf:	Standard Gibbs free energy of formation
hf:	Enthalpy of formation at standard conditions
hfus:	Enthalpy of fusion at standard conditions
hvap:	Enthalpy of vaporization at standard conditions
log10ws:	Log10 of Water solubility in mol/l
logp:	Octanol/Water partition coefficient
mcvol:	McGowan's characteristic volume
pc:	Critical Pressure
tb:	Normal Boiling Point Temperature
tc:	Critical Temperature
tf:	Normal melting (fusion) point
vc:	Critical Volume

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CASE NOTES

Clobenzorex/ Amphetamine / Methamphetamine Death in L.A.

Submitted by Daniel Anderson, Los Angeles County Department of the Coroner

Professional jockey found unresponsive at home with numerous pills, including Clobenzorex.

Toxicology results (partial)-ug/ml or ug/g

	Clobenzorex	Amphetamine	Methamphetamine
Heart Blood	1.9	3.1	0.84
Femoral Blood	QNS	1.3	0.50
Bile	14	15	4.7
Liver	174	15	2.6
Gastric	4.9 mg	0.22 mg	0.12 mg
Urine	1.3	2.0	4.8

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Valtier S, Cody JT. Metabolic production of amphetamine following administration of clobenzorex. J Forensic Sci 1999;44(1):17-22.

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Valtier S, Cody JT. A Gas Chromatography-Mass Spectrometry Method for the Quantitation of Clobenzorex. J Anal Tox, 1999;23: 603-608.

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ANNOUNCEMENT

"CASE NOTES" FEATURED IN NEXT ISSUE

The June 2001 Issue of ToxTalk will be dedicated to Case Reports. Please adhere to the usual **ToxTalk** deadline for this issue, May 1, and submit your contributions to:

Ines B. Collison, Ph.D., Forensic Scientist
Orange County Sheriff-Coroner Department
320 N. Flower, Santa Ana, CA 92703
e-mail: rd329@fss.co.orange.ca.us Tel: 714-834-6402 Fax: 714-834-4519

Remember, a typical case note is only 1/2 to a full page.

Cocaine and Marijuana Monographs Now Available!

Web Master Bruce Goldberger has announced the cocaine and marijuana monographs have recently been posted. To gain access to SOFT's on-line membership directory and drugs and driving pages, you must type the username and password. USERNAME: drugs PASSWORD: #cocaine!

Visit the SOFT web-site at www.soft-tox.org and get the latest information on items of interest to SOFT members.

Visit the SOFT Web-site at www.soft-tox.org

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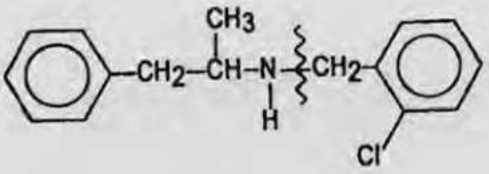
Clobenzorex (Asenlix®, Dinintel®, Finedal®)

General

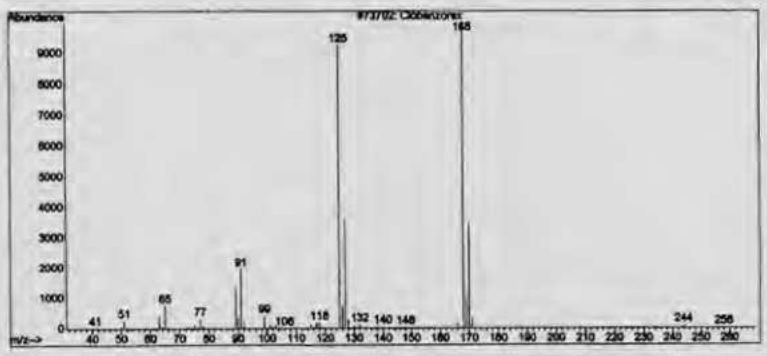
Clobenzorex is a relatively new anorectic drug that has been cited in the literature the last couple of years. It is not available in the United States, but is available in other countries such as Mexico, Spain, Argentina, and France. Clobenzorex is manufactured by Hoechst Marion Roussel as a 30 mg capsule, dark/light green in color and marked with HMR. Clobenzorex metabolizes to d-amphetamine and has amphetamine-like central stimulant behavioral properties.

Chemical

- Clobenzorex Hydrochloride
  - (+)-N-(2-Chlorobenzyl)-alpha-methylphenethylamine hydrochloride
  - $C_{16}H_{18}ClN$-HCl
  - Formula weight 296.2
  - Molecular weight 259.5
  - CAS Registry No. 13364-32-4 (Clobenzorex), 5843-53-8 (Clobenzorex HCl)



- Clobenzorex is a basic drug and can be extracted with an n-butylchloride liquid/liquid extraction and an acid back extraction. According to the literature, it also can be extracted and derivitized very similar to amphetamine/methamphetamine-type compounds.
- Detection of clobenzorex is possible by either GC/NPD or GC/MS.



Ions: 168, 125, 170, 127, 91, 244, 258 m/z

Relative retention time of Clobenzorex (0.8558/Carbinoxamine) to other commonly encountered basic drugs are as follows:

Phencylcidine, Doxylamine, Tramadol, **CLOBENZOREX**, Chlorpheniramine, Cyclizine, Metoprolol

Pharmacology

- Symptoms include dry mouth, significant excitation, increased alertness and difficulty sleeping.
- Approximately 20 times less potent than (+)amphetamine
- 20% of a dose can be accounted for (after 24 hrs) as d-amphetamine or amphetamine metabolites.
- Principal metabolites excreted in urine of rats: 40% glucuronide, 5% amphetamine, 3% p-hydroxyamphetamine.
- Clinical studies

	Urine C_{max} (ug/ml)	
	Clobenzorex	Amphetamine
Single 30 mg dose	0.029	2.47 - Detectable for up to 116 hours post dose
30 mg dose for 7 days	0.047	4.7

Editor's note: See Case Notes "Clobenzorex / Amphetamine / Methamphetamine Death in L.A."

NEW DRUGS is making its debut in this issue of ToxTalk. Editor Joseph Monforte would like to know what you think of this feature. Please direct your comments to: DrMonforte@aol.com

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Clobenzorex

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Various toxicites secondary to clobenzorex addiction: case report

A 22-year-old man developed dilated cardiomyopathy, heart failure, acute pulmonary oedema, cardiomegaly and left pulmonary hypertrophy following clobenzorex addiction [*indication not stated*].

The man was occasionally consuming alcohol in moderate quantities with the regular oral and nasal consumption of amphetamine-type stimulants and cocaine during the last three years in a non-specified quantity. Two weeks prior to the hospitalisation, on 14 April 2016, he developed fits of dry cough, which became humid during the following week. A sharp pain appeared in the right hemithorax which was exacerbated by drawing breath. He reported consumption of clobenzorex 60mg 12 tablets (total dose of 720mg). Eight hours later, he presented with two episodes of vomiting, sudden increase in the intensity and duration of the fits of coughing and sudden-onset dyspnoea of minimal efforts, rapidly progressing to be symptomatic at rest. He also had orthopnea and required support by four pillows to be comfortable. Subsequently, due to all of this, he presented to an emergency department. He presented with tachycardia and was polypneic with a respiratory frequency of 24 /minute. The blood oxygen saturation with no supplementary oxygen was 84%, which increased with an oxygen mask with supplementary oxygen. Physical exploration showed bilateral crackles at the pulmonary auscultation. A chest X-ray revealed bilateral diffuse opacities along with cardiomegaly grade III. He was admitted with an initial diagnosis of pneumonia.

The man started receiving antibiotic therapy. On day 3, he presented with a sudden severe worsening of his clinical condition, with increased polypnoea, tachycardia, dizziness and chest pain. The non-invasive mechanical ventilation was required and he was transferred to the ICU. An ECG revealed left ventricular hypertrophy. Acute pulmonary oedema was noted in thorax CAT scan. A dilated cardiomyopathy was suspected. An ECG revealed depressed ejection fraction of 19%, along with severe generalised hypokinesis. He was treated for heart failure with levosimendan and diuretics. In the following week, a significant clinical improvement was noted and he was transferred to the Internal Medicine Ward. On the last day of hospital, his vitals were stable and he had no fever or dyspnoea.

Author comment: "*In the following case report we present a 22 years old patient with several risk factors associated with cardiomyopathy, the main one we purport to be a chronic use of amphetamines type stimulants, which presented to the emergency department with acute onset dyspnea.*" "*Its cardiotoxic effect is thought to be due to exposure of the myocardium to excessive catecholamine levels, and amphetamine-inherent cardiotoxicity.*"

Solis-Olivares CA, et al. Amphetamine-related dilatated cardiomyopathy: A growing phenomenon. Case report. Revista Mexicana de Cardiologia 28: 35-39, No. 1, Jan-Mar 2017. Available from: URL: http://www.medigraphic.com/pdfs/cardio/h-2017/h171e.pdf - Mexico

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disease. Physical examination revealed a palpable liver, 8cm below the right costal margin. Laboratory studies showed: AST 53 U/L, ALT 41 U/L, alkaline phosphatase 238 mg/dl, alpha–fetoprotein 2.5 ng/ml, calcium 12.7 mg/dl, phosphorus 6.36 mg/dl. Abdominal CT scan revealed a 14 x 12 x 10 cm low–density mass in the right lobe of liver. MRI showed on T1–weigthed images a hypointense hepatic mass; on the T2–weigthed images hyperintensity was evident. At surgical exploration a large liver mass was detected, occupying the entire right lobe. A right trisegmentectomy was performed with tumor grossly resected with microscopic residual disease (i.e. positive margins). On microscopic examination, the tumor was composed mainly of two components which were intermingled; epithelial and mesenchymal elements; the diagnosis was mixed hepatoblastoma. The postoperative course was uneventful , and calcium level dropped to 7.8 mg/dl. The patient is alive, and received 3 cycles of systemic chemotherapy with cisplatinum an adryamicin.

Discussion.: Hepatoblastomas accounts for 0.2–5.8% of total malignancies of the liver. Hepatoblastomas in adults is extremely rare and the prognosis is poor because they are usually discovered late. Absence of elevated alpha–fetoprotein may be a poor prognostic sign, as reported previously. Occasionally may produce paraneoplastic manifestations such as production of beta–human chorionic gonadotropin. Hypercalcemia as in this case, was not reported previously. According to the postsurgical staging our patient was in a stage II. In children with stage I and II, surgical resection and systemic chemotherapy have a cure rate of 60–90% approximately. Because the lack of experience of hepatoblastomas in adult patients, we think that those tumors can be treated successfully with surgical resection (if it is possible) and chemotherapy as in children.

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GASTROINTESTINAL HEMORRHAGE FROM AN ILEAL POLYP

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Introduction: Massive bleeding from the small intestine is uncommon and difficult to localize. We recently encountered a case of severe gastrointestinal hemorrhage from an ileal adenoma.

Case Report: A 70–yr–old male with chronic atrial fibrillation and history of aortic valve replacement, on warfarin, presented to the emergency room after several episodes of black tarry stools followed by one episode of frank hematochezia over 2 days. He denied use of NSAIDs. He did not smoke or drink alcohol. His blood pressure was 50/ 20 mm Hg, Hgb 9.6 g/dl and INR 3.9.

The patient was volume resuscitated, anticoagulation was reversed and endoscopy was performed in 24 hours. Upper endoscopy was normal. Colonoscopy showed blood clots throughout the colon, no bleeding source identified. Ileoscopy performed at the time of colonoscopy revealed fresh blood filling the ileum. Tagged red blood cell scintigraphy revealed increased activity in the right lower quadrant in the area of terminal ileum and cecum. The patient continued to bleed, requiring multiple transfusions, and underwent exploratory laparotomy on day 3 of admission. A bleeding polyp was identified in the terminal ileum; right hemicolectomy with terminal ileal resection and reanastomosis was done. The surgical specimen revealed a broad based sessile polyp measuring 3 x 1.5 x 2cm, 18 cms proximal to the ileocecal valve with a fresh blood clot adherent to the surface. Histology was villous adenoma with focal high–grade dysplasia on the surface. The patient had an uneventful postoperative course.

Conclusion: Small bowel benign polyps are uncommon and can rarely cause severe gastrointestinal bleeding. A deep ileoscopy should be attempted at the time of colonoscopy in all patients with unexplained gastrointestinal bleeding. Management may require a combination of diagnostic modalities and surgery for definitive therapy (1).

References: 1. Eisen GM, et. al. An annotated algorithmic approach to acute lower gastrointestinal bleeding. Gastrointest Endosc. 2001 Jun;53(7): 859–63.

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CLOBENZOREX–INDUCED HEPATITIS: A CASE REPORT FROM US–MEXICO BORDER

Tong Jing, M.D., Saket Prasad, M.D., Armando D. Meza, M.D. and Joshua Demke, B.S. Internal Medicine, Texas Tech University Health Sciences Center, El Paso, TX.*

Purpose: Clobenzorex hydrochloride (ASENLIX®) manufactured by Roussel (Mexico) is metabolized to amphetamine and has been used as an anorexic agent in the treatment of obesity. Although not sold in the United States, it is readily available in Mexico and it is one of the top 15 drug products that U.S. residents traveling across the border to purchase. Little is known about its potential adverse effect, we report a case of severe clobenzorex–induced hepatitis.

Case: A 38–year old, female, with no significant past medical history, present with right upper quadrant abdominal pain for one week. The pain was constant, sharp, and four out of ten intensity, radiated to the back, associated with nausea, vomiting, generalized myalgia, prutitus, and yellow discoloration of sclera. Three weeks prior to admission, she started self medicating with clobenzorex, one tablet (30 mg) every other day taking a total of seven tablets over a two week period and discontinuing it secondary to nausea. She had recently purchased this in Juarez (Mexico) to use as a weight–control agent. Physical examination revealed an obese female with severe jaundice, mild right upper quadrant abdominal tenderness, but no hepatomegaly or splenomegaly. Laboratory studies revealed a total bilirubin 20.1 mg/dL with direct bilirubin more than 10.0 mg/dL, AST 3100 IU/L, ALT 3270 IU/L, alkaline phosphatase 169 IU/dL, albumin 3.1 g/dL, PT 14.2 s, PTT 28.2 s. CBC was normal. Hepatitis A, B and C serologies were negative. ANA and AMA were negative. Thyroid function tests were normal. Abdominal ultrasound showed a normal liver and biliary tree. Whilst remaining clinically stable, her liver enzymes continued to remain elevated and liver biopsy was done on day three. It showed the portal areas predominantly infiltrated with neutrophils, the liver parenchyma revealed extensive ballooning degeneration and necrosis of liver cells with intracytoplasmic bile stasis, which were consistent with drug–induced hepatitis. She was given supportive and symptomatic treatment, discharged on day eight once liver enzymes started to improve. Her symptoms resolved with normalization of liver chemistries over a period of six weeks.

Conclusion: 1) To our knowledge, this is the first case report of clobenzorex induced acute hepatitis. 2) When clinician encounter a patient with clinical picture of acute hepatitis, detailed medication and travel history

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including the nonprescription medication is very important. 3) The public needs to be aware of dangers of self–medicating with drug products from across the US–Mexico border.

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BILIARY PAPILLOMATOSIS TREATED WITH INTRADUCTAL PHOTODYNAMIC THERAPY

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Purpose: We describe a case of diffuse biliary papillomatosis (BP) treated with photodynamic therapy (PDT).
Methods: An 86–year–old male presented with painless jaundice. Labs revealed: Total Br 19.1 mg/dL (direct 13.3), Alk phos 1016 U/L and CA 19–9 459 (normal < 40). CT showed marked dilatation of the intra– and extra–hepatic bile ducts. EUS revealed a dilated CBD with an infiltrative lesion extending into both right and left hepatic ducts and the intrahepatic biliary tree. ERCP with cholangioscopy and intraductal ultrasound revealed large amounts of intraductal mucus, markedly thickened mucosa with widespread papillary projections and a normal appearing pancreatic duct. Biliary mucosal biopsies revealed papillary epithelium with low–grade dysplasia, consistent with BP.
Results: Given the patient's age and reluctance to undergo surgical resection, intraductal PDT was performed with a total of 130 joules/cm2 of bile duct surface area using a 2.5 cm cylindrical diffusing power at a power output of 1.0 watts per centimeter fiber. At follow–up, six weeks later, the patient was asymptomatic and labs showed marked improvement with total serum Br of 4.7, direct Br 2.7.

BP is a exceedingly rare entity characterized by multicentric papillary lesions of intra and extrahepatic biliary epithelium with varying degrees of dysplasia. It is difficult to treat because of its diffuse nature, high rate of recurrence and malignant potential. Treatment options include surgery or endoscopic evacuation of mucus with stenting. Other reported modalities include liver transplantation in two cases and intraluminal brachytherapy in one. PDT is based on the selective accumulation of a photosensitizer in malignant tissues and the subsequent irradiation with laser light. Its current spectrum of use includes malignant (esophageal cancer) and non–malignant (actinic keratosis) conditions. It has been reported to palliate patients with cholangiocarcinoma. Reported complications include photosensitivity, pain, and stricutres. PDT did not result in any complications in our patient.
Conclusions: PDT may be a less invasive alternative for palliation of biliary obstruction in these patients. The application of PDT appears to be safe, and studies with longer durations of follow–up are needed to fully evaluate the safety and efficacy of this procedure in BP. This is the first reported case to our knowledge of PDT in BP.

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POSTOPERATIVE LIVER ENZYME ABNORMALITES IN AN IMMUNOSUPPRESSED ADULT

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Purpose: We describe an unusual cause of hepatitis in an immunocompromised patient
Report of a Case: A 71–year–old woman developed abnormal laboratory tests after surgical resection for jejunal diverticulitis. She had been on prednisone for three months for giant cell arteritis. Preoperative liver enzymes were normal. Eight days after jejunal resection she developed fever, right upper quadrant abdominal pain, somnolence, ALT 3500 U/L, AST 9000 U/L, total bilirubin 0.5 mg/dL and prothrombin time 18 seconds. Her leucocyte count was normal and tests for acute hepatitis A, B and C were negative. There were no reported hypotensive episodes perioperatively. Ultrasound of the abdomen with doppler was normal. Repeat liver enzymes the following day were unchanged. Liver biopsy demonstrated that the acinar parenchyma was widely involved with nonzonal hemorrhagic necrosis. Hepatocytes adjacent to the necrosis had ground glass–like nuclear inclusions consistent with herpes and in situ hybridization was positive for herpes. She was treated earlier with acyclovir; however, a day later she progressed to stage four–encephalopathy with an ammonia level of 200 ugN/dL and an intracranial pressure monitor was placed. Her intracranial pressure was normal. She was treated with lactulose with normalization of ammonia. In addition, liver enzymes improved; however, the patient developed multiple seizures. Imaging studies of the head demonstrated changes consistent with watershed infarcts with no evidence for encephalitis or cerebral vasculitis. EEGs showed persistent multiple focal motor seizure activity despite treatment with phenytoin and lorazepam. Moreover, she became septic and developed hypotension. Because of the low chance of central nervous system recovery, support was withdrawn and the patient died. An autopsy was refused.
Conclusions: Herpes virus is an unusual cause of hepatitis but should be considered in immunosuppressed patients with very high aminotransferases. The definitive diagnosis requires liver biopsy. Early treatment can improve liver function but overall survival remains poor.

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GRAFT VERSUS HOST DISEASE AND POST TRANSPLANT LYMPHOPROLIFERATIVE DISORDER OCCURRING IN THE SAME PATIENT AFTER ORTHOTOPIC LIVER TRANSPLANTATION

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Purpose: We describe an unusual case of both graft versus host disease (GvHD) and post transplant lymphoproliferative disorder (PTLD) that developed in a patient following orthotopic liver transplantation (OLT).
Report of a Case: A 49–year–old woman developed fever, sore throat and a generalized non–pruritic rash one month after liver transplantation for primary biliary cirrhosis. Her immunosuppression consisted of tacrolimus, mycophenolate mofetil and prednisone. Blood cultures and viral serologies were negative. Her leukocyte count was 3000/mm^3 with a platelet count 60x10^3/mm^3 and a hemoglobin of 8g/dL. Liver enzymes and an ultrasound of the abdomen with doppler were normal. Multiple skin biopsies were noteworthy for lichenoid dermatitis. She subsequently developed melena. Upper endoscopy revealed proximal esophageal ulcerations and an esophageal biopsy demonstrated individual cell apoptosis and focal epithelial cell vacuolization between the epidermis and lamina propria consistent with GvHD. Immunostain for herpes virus was negative. Serological HLA class I typing demonstrated 50% of circulating immunocytes were donor derived. A mediastinal lymph node biopsy demonstrated PTLD with a monoclonal, diffuse large B–cell lymphoma and EBV positive cells. Immunosuppressive medications were discontinued and she was treated with anti CD 20 monoclonal antibody (Rituxan®) 375mg/m^2/wk for four weeks pending a follow up CT scan of the chest. In addition, her skin rash has progressed to erythroderma with vesicle formation and she continues to have fever and diarrhea despite negative blood and stool cultures.
Conclusions: GvHD has become increasingly recognized after liver transplantation. This is the first known case of concurrent GvHD and PTLD occurring in a patient after orthotopic liver transplantation.

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GASTRODUODENAL INVOLVEMENT IN PATIENTS WITH ULCERATIVE COLITIS

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Journal of Chromatography B, 689 (1997) 81–89

JOURNAL OF CHROMATOGRAPHY B

Gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) in toxicological analysis
Studies on the detection of clobenzorex and its metabolites within a systematic toxicological analysis procedure by GC–MS and by immunoassay and studies on the detection of α- and β-amanitin in urine by atmospheric pressure ionization electrospray LC–MS

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Abstract

GC–MS is the method of choice for toxicological analysis of toxicants volatile in GC while non-volatile and/or thermally labile toxicants need LC–MS for their determination. Studies are presented on the toxicological detection of the amphetamine-like anorectic clobenzorex in urine by GC–MS after acid hydrolysis, extraction and acetylation and by fluorescence polarization immunoassay (FPIA, TDx (meth)amphetamine II). After ingestion of 60 mg of clobenzorex, the parent compound and/or its metabolites could be detected by GC–MS for up to 84 h or by FPIA for up to 60 h. Since clobenzorex shows no cross-reactivity with the used immunoassay, the N-dealkylated metabolite amphetamine is responsible for the positive TDx results. The intake of clobenzorex instead of amphetamine can be differentiated by GC–MS detection of hydroxyclobenzorex which is detectable for at least as long as amphetamine. In addition, the described GC–MS procedure allows the simultaneous detection of most of the toxicologically relevant drugs. Furthermore, studies are described on the atmospheric pressure ionization electrospray LC–MS detection of α- and β-amanitin, toxic peptides of amanita mushrooms, in urine after solid-phase extraction on RP-18 columns. Using the single ion monitoring mode with the ions m/z 919 and 920 the amanitins could be detected down to 10 ng/ml of urine which allows us to diagnose intoxications with amanita mushrooms.

Keywords: Clobenzorex; Amanitin; Amphetamine; Toxicological analysis

1. Introduction

Clinical toxicological analysis is usually a single-case analysis which must be available on a 24-h

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basis. The method should be as rapid and precise as necessary for clinical diagnosis and therapy. Ideally, one single procedure should allow the detection of all relevant potential toxicants. Forensic toxicological analysis should also provide high reliability and accuracy in order to be defensible in court. Therefore, GC–MS is the method of choice for detection and quantification of toxicants volatile under GC conditions, whereas non-volatile toxicants require LC–MS.

The first step of the toxicological analysis is the identification of the usually unknown drugs and poisons in body fluids. A systematic toxicological analysis (STA) procedure is necessary that allows the simultaneous detection of as many toxicants as possible in biosamples. Unspecific chromatographic methods, even if several were combined [1], proved to be insufficient for these purposes, because merely the same chromatographic behavior of two compounds is not evident for their identity [2]. Besides, all chromatographic peaks or spots have to be identified because any of them may represent a potential poison. An STA procedure using GC–MS was developed that allows the simultaneous screening and identification of most of the toxicologically relevant drugs and their metabolites [3]. New drugs or poisons should be integrated in this STA procedure.

Clobenzorex [*S*-(+)-N-(2-chlorobenzyl)-amphetamine] is a widely used anorectic which is metabolized to the pharmacologically active *S*-(+)-amphetamine [4]. For two reasons, it is of clinical and forensic interest: abuse of clobenzorex as a stimulant is known and therapeutic use of clobenzorex may mislead to suspect amphetamine abuse when urine samples of the patients are tested for drug abuse [5]. Therefore, according to the standard guidelines in toxicological analysis a screening and a confirmation procedure are necessary for detection of an abuse or for differentiation of a therapeutic use of clobenzorex from an amphetamine abuse. A GC–MS procedure for the detection of sympathomimetic drugs [6] only allows the detection of clobenzorex itself, while detection of its metabolites is necessary for differentiation in the later phase of excretion. In the following, studies are presented on the toxicological detection of clobenzorex and its metabolites in urine by GC–MS and fluorescence polarization immunoassay (FPIA) and on the duration of detectability by these procedures.

As mentioned above, non-volatile toxicants cannot be detected by GC–MS. Therefore, we are developing LC–MS procedures using an atmospheric pressure ionization electrospray interface for those compounds. Among the non-volatiles, α- and β-amanitin, toxic peptides of amanita mushrooms, show the greatest relevance in clinical toxicology. Highly specific detection of amanitins in body fluids is necessary for an early diagnosis of an intoxication entailing a large scale of invasive and expansive therapy. In 1992 Dorizzi et al. [7] have critically reviewed methods published for the determination of amatoxins in biological matrices. Determination of amanitins in urine by a commercially available radioimmunoassay (RIA) is possible but the tracer is only stable for 1–2 months and the assay is not available throughout the year. Moreover, interferences with urine matrix are possible so that confirmation of the RIA results by alternative techniques is required [7]. Several of the reviewed HPLC methods are suitable for separation of the amanitins. However, electrochemical or UV detection requires a complex protocol of extraction and purification steps often followed by column switching techniques. Therefore, we have developed a LC–MS method for the detection of α- and β-amanitin in human urine after a simple solid-phase extraction.

2. Experimental

2.1. Detection of clobenzorex and its metabolites in urine by GC–MS and FPIA

2.1.1. Chemicals and reagents

All chemicals used were obtained from E. Merck (Darmstadt, Germany) and were of analytical grade. Clobenzorex was obtained from Roussel Uclaf (Paris, France).

2.1.2. Urine samples for GC–MS

After informing them according to the declaration of Helsinki and obtaining written consent, three healthy volunteers received a single oral dose of 60 mg of clobenzorex. Urine samples were collected every 4 h for 8 days. All samples were stored at

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−20°C before analysis. Blank urine samples were collected before drug administration to check whether the samples were free of interfering compounds.

2.1.3. Sample preparation of urine for GC–MS

A 10-ml volume of urine was refluxed with 3 ml of 30% hydrochloric acid for 15 min. Following hydrolysis, the sample was basified with 4 ml of 10 mol/l aqueous sodium hydroxide and the resulting solution was mixed with 10 ml of 30% aqueous ammonium sulphate to obtain a pH between 8 and 9. This solution was extracted with 10 ml of a dichloromethane–isopropanol–ethyl acetate mixture (1:1:3, v/v). After phase separation by centrifugation (5 min, 1500 *g*), the organic layer was evaporated to dryness. The residue was derivatized by acetylation with 100 μl of acetic anhydride–pyridine (3:2, v/v) for 30 min at 60°C. After evaporation of the derivatization mixture, the residue was dissolved in 100 μl of methanol and 0.5–1 μl were injected into the gas chromatograph.

2.1.4. Gas chromatography–mass spectrometry

Clobenzorex and its metabolites were separated and identified in the acetylated urine extracts using a Hewlett-Packard (HP, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP MS Engine 5989 Series A mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software. The GC conditions were as follows: splitless injection mode; column, HP capillary (12 m× 0.2 mm I.D.), cross linked methylsilicone, 330 nm film thickness; injection port temperature, 280°C, carrier gas, helium; flow-rate, 1.9 ml/min; column temperature, programmed from 100–310°C at 30°C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode; ionization energy, 70 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C. For detection of clobenzorex and its metabolites, mass chromatography with the selected ions *m/z* 86, 118, 168 and 170 was used. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu which executes the user defined macros (the macros can be obtained from the authors). The identity of the peaks in the mass chromatograms was confirmed by computer library search of the underlying mass spectra [8].

2.1.5. Fluorescence polarization immunoassays (FPIA)

The urine samples from the volunteers were used for immunological determination. The TDx system of Abbott (Irving, TX, USA) with the amphetamine–methamphetamine II assay (AM/MA II) was applied. The cut-off value and the detection limit in urine recommended by the manufacturer were 300 and 100 ng/ml, respectively. Blank urine samples were spiked with clobenzorex at a concentration of 8000 ng/ml for determination of its cross-reactivity with the assay.

2.2. Detection of α- and β-amanitin in urine by LC–MS

2.2.1. Chemicals and reagents

Erythromycin and α- and β-amanitin were obtained from Sigma-Aldrich (Deisenhofen, Germany). Methanol, water (both HPLC grade), ammonium acetate, acetic acid (both analytical grade) and the LiChrolut RP-18 (500 mg) cartridges for the solid-phase extraction (SPE) were obtained from E. Merck.

2.2.2. Urine samples for LC–MS

Blank urine samples were collected from healthy volunteers. Authentic urine samples were obtained from patients recently intoxicated by amanita mushrooms.

2.2.3. Sample preparation of urine for LC–MS

A 5-ml volume of urine was first diluted 1:1 with distilled water. The SPE columns were washed with ten reservoir volumes of aqueous acetic acid (1%), activated with 3 ml of methanol and rinsed with 3 ml of water. The sample was loaded onto the column, washed with 3 ml of aqueous acetic acid (1%) and then eluted with 6 ml of methanol. The eluate was evaporated to dryness at 65°C. The residue was redissolved in 100 μl of mobile phase and 20 μl were injected into the HPLC system with complete loop filling mode.

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2.2.4. Liquid chromatography–mass spectrometry

The amanita toxins α- and β-amanitin were separated and identified in urine extracts using a Hewlett-Packard API-ES LC–MS including an electrospray interface (HP 59987A) and an HP-MS Engine 5989 Series B. The MS conditions were as follows: single ion monitoring (SIM) mode with the ions *m/z* 919 and 920 for α- and β-amanitin, respectively. Tuning of the MS was performed in three steps: first tuning with an erythromycin solution (100 μg/ml of methanol–water (1:1, v/v), tuning ions *m/z* 558, 716 and 734). Fine tuning with a β-amanitin solution (10 μg/ml of mobile phase, tuning ion *m/z* 920). These two tuning steps were done using a syringe pump (Harvard, South Natick, MA, USA) at a flow-rate of 75 μl/min. Last performance check by injection of a standard solution of α- and β-amanitin (5 μg/ml of mobile phase, respectively) into the LC–MS under normal LC–MS conditions. A Valco six-port injection valve was used to connect either the syringe pump or the HPLC system to the electrospray chamber. The HPLC consisted of an HP 1050 equipment with a manual injector, a 20-μl sample loop and a variable-wavelength detector and an electrospray interface. The isocratic separation was achieved on a Chromcart CC 125/2 Kromasil RP-18 narrowbore column (125×2 mm I.D.) with 5 μm particle size and a Kromasil guard column from Macherey-Nagel (Düren, Germany). The HPLC mobile phase was a 22:78 mixture (v/v) of methanol–ammonium acetate (0.02 mol/l, adjusted to pH 5). Before use, the mixture was degassed for 30 min in an ultrasonic bath. During use, the mobile phase was degassed with helium. The flow-rate for the LC–MS was 75 μl/min. UV detection was performed at 302 nm before the effluent entered the electrospray interface.

3. Results and discussion

3.1. Detection of clobenzorex and its metabolites in urine by GC–MS within a STA procedure and by FPIA.

3.1.1. Sample preparation

For sample preparation, a procedure was used that has proved to be successful for systematic toxicological analysis [3]. Since the hydroxy metabolites of clobenzorex are excreted as conjugates and hydroxyclobenzorex is the main metabolite, rapid acid hydrolysis was performed before isolation and derivatization. Derivatization was essential for sensitive detection of the polar clobenzorex metabolites. Acetylation has been approved for the identification of numerous drugs and their metabolites as described in review articles [3,9]. It leads to stable derivatives with good gas chromatographic properties. The acetylation mixture can be evaporated before analysis so that the resolution power of capillary columns does not decrease in contrast to other derivatization reagents. The molecular mass does not increase very much, in contrast to silylation or perfluoroacylation, so that compounds with relatively high molecular mass and several derivatizable groups can be measured with low-priced mass selective detectors with a mass range only up to 650 u. The analytical recovery of clobenzorex and its metabolite amphetamine determined at urine concentrations of 1000 ng/ml was 85±10 and 57±6%, respectively.

3.1.2. Detection by GC–MS

The full mass spectra recorded during temperature-programmed GC were evaluated using mass chromatography. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu (Fig. 1) which executes the user-defined macros. The selected ions *m/z* 86, 118, 168, 170 were used for indicating the presence of acetylated clobenzorex and its metabolites in urine: amphetamine (86, 118; peaks 1 in Fig. 1), norephedrine and hydroxyamphetamine (86; peaks 2–3 in Fig. 1) or clobenzorex and its non-dealkylated metabolites (168, 170; peaks 4–7 in Fig. 1). Clobenzorex itself is not indicated in Fig. 1, because this urine sample was taken at a time when clobenzorex was no longer being excreted (20 h after ingestion). The identity of the peaks in the mass chromatograms was confirmed by computerized comparison of the underlying mass spectra with reference spectra recorded during this study [8]. The reference spectra, the gas chromatographic retention indices (RI) and the structures of acetylated clobenzorex and its main metabolites are shown in Fig. 2. The numbers of the spectra correspond to those of the peaks in Fig. 1. The retention indices were recorded during the GC–MS procedure (Section 2.1.4.) and calculated in

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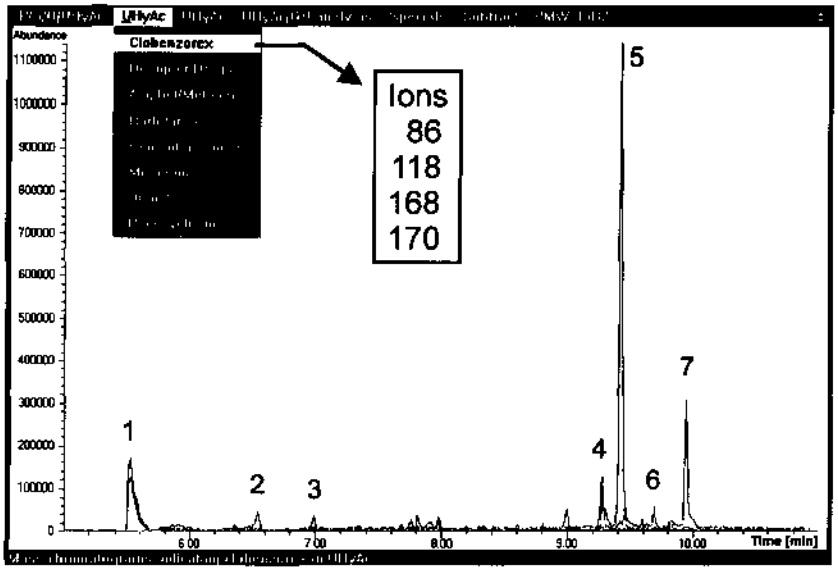

Fig. 1. Typical mass chromatograms with the ions m/z 86, 118, 168 and 170 indicating the presence of acetylated clobenzorex metabolites in urine 20 h after ingestion: amphetamine (1), norephedrine (2), hydroxyamphetamine (3), isomer 1 of hydroxyclobenzorex (4), isomer 2 of hydroxyclobenzorex (5), hydroxymethoxyclobenzorex (6) and dihydroxyclobenzorex (7). The merged mass chromatograms can be differentiated by their colours on a colour screen.

correlation with the Kovats' indices [10] of the components of a standard solution of typical drugs which was measured daily for testing the GC–MS performance [11,12]. The minor metabolites of clobenzorex are not essential for the screening procedure. As given in the legend to the spectra, the clobenzorex metabolites amphetamine, hydroxy amphetamine and norephedrine are either drugs or common metabolites of further stimulants or anorectics. However, the detection of the non-dealkylated metabolites, especially hydroxyclobenzorex (spectrum no. 5 in Fig. 2) allowed the differentiation of the drugs. If racemic amphetamine, commonly used as a street drug, was taken in addition, differentiation from the clobenzorex metabolite *S*-(+)-amphetamine could be performed by enantioselective detection [13]. Interferences by biomolecules or further drugs could be excluded because of mass spectral identification. The mass spectra of all the endogenous biomolecules detectable using the described procedure are included in our library and handbook [8,14]. The limit of detection in urine was 50 ng/ml of clobenzorex and 100 ng/ml of the metabolite amphetamine ($S/N=3$).

3.1.3. Detection by immunoassay

Clobenzorex shows no cross reactivity with the AM/MA II assay because of the large chlorobenzyl substituent in the side chain. Even at the high concentration level tested (8000 ng/ml) the measured values were not higher than those of the blank urine samples. For lack of reference substance of the main metabolite (conjugated) hydroxyclobenzorex, its cross reactivity could not be determined. Due to the intact side chain it can be concluded that it also shows no cross reactivity with the TDx AM/MA II. Since clobenzorex is metabolized to amphetamine the assay gives positive results in urine after ingestion of clobenzorex. The TDx values measured during our excretion study ranged between 100 and 10 000 ng/ml (cross reactivity value of amphetamine: 36% at a concentration level of 1000 ng/ml).

3.1.4. Duration of detectability of clobenzorex and its metabolites in urine by GC–MS and FPIA

The duration of detectability of clobenzorex and its metabolites in urine by GC–MS and FPIA is shown in Fig. 3. After a single oral dose of 60 mg of clobenzorex the AM/MA II assay gave positive

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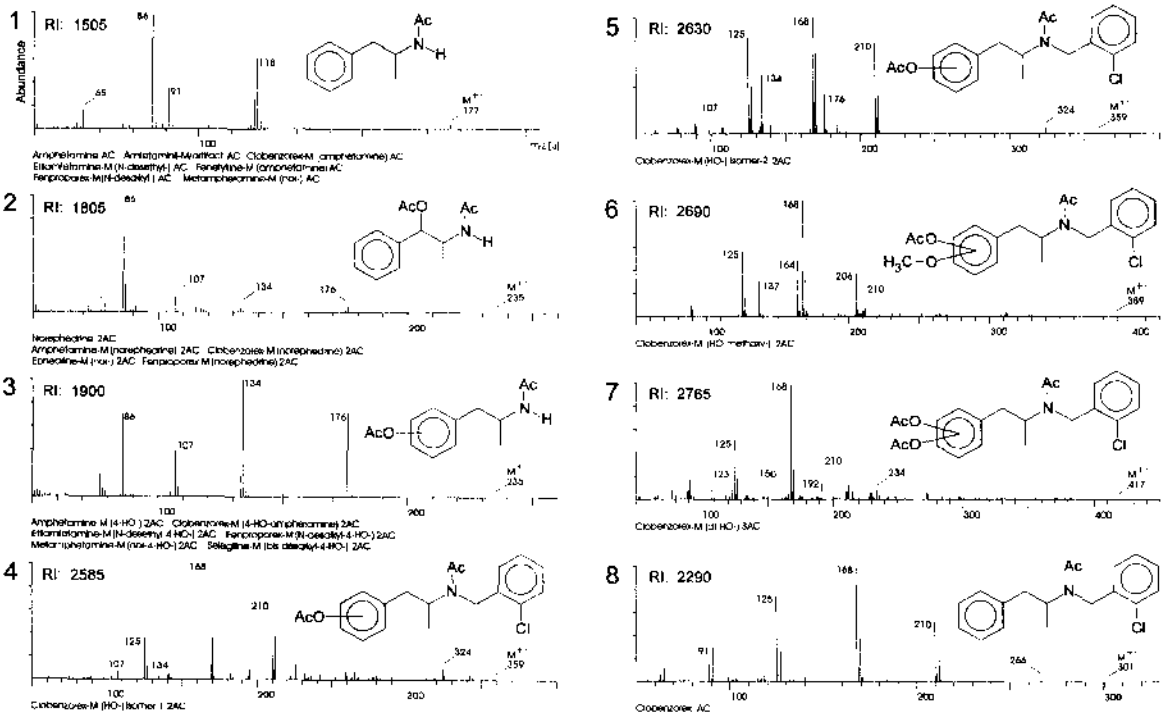

Fig. 2. Mass spectra, GC retention indices (RI) and structures of acetylated (Ac) clobenzorex and its main metabolites for precise identification. All the molecular ions (M^+) are present with low abundance. The numbers of the spectra correspond to those of the peaks in Fig. 1.

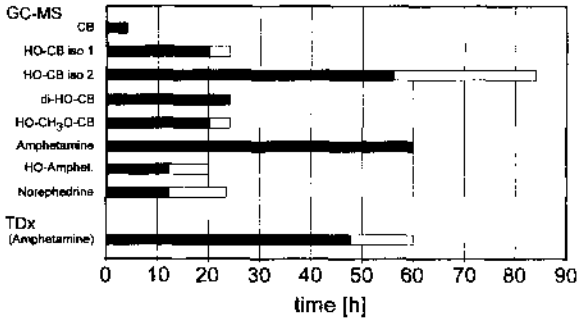

Fig. 3. Duration of detectability of clobenzorex and its main metabolites (hours after ingestion) by the TDx assay AM–MA II and GC–MS in urine samples after ingestion of 60 mg of clobenzorex ($n=3$).

results in urine for 40 to 60 h taking into consideration the cut-off value. The following metabolites could be detected in urine by the described GC–MS procedure during the given time: unchanged clobenzorex (CB) for only 4 h, hydroxy clobenzorex (HO–CB) for 56 to 84 h and amphetamine for about 60 h. The other metabolites described here were detectable for 12 to 24 h. All the immunological results could be confirmed by GC–MS detection of amphetamine (Fig. 3). The intake of clobenzorex instead of amphetamine can be differentiated by detection of hydroxyclobenzorex which is detectable at least as long as amphetamine. However, it should be stated clearly that cleavage of conjugates and derivatization are indispensable for the long term detection of hydroxyclobenzorex. Detection of unchanged clobenzorex recommended by Franceschini et al. [6] is not suitable because clobenzorex is only detectable in minor amounts within the first few hours after ingestion while amphetamine is detectable for up to

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40 to 60 h. The variation in the time of excretion of clobenzorex may be caused by inter-individual differences e.g. relative body mass and/or renal function.

3.2. Detection of α- and β-amanitin in urine by LC–MS

3.2.1. Sample preparation for LC–MS

SPE has proved to be suitable for the extraction of α- and β-amanitin from biological matrices [7]. The Merck LiChrolut RP-18 columns used gave better results than several other tested columns. The ten times washing of the cartridges with 1% aqueous acetic acid led to more reproducible results. Addition of acetic acid to the washing solution was necessary to prevent the carboxy group of β-amanitin from deprotonation and elution from the column during the washing step. Concentration by a factor of 50 was necessary to improve the detection at low concentrations. The analytical recovery of α- and β-amanitin determined at urine concentrations of 100 ng/ml was 65±7 and 63±5%, respectively.

3.2.2. Detection by LC–MS

The coupling of HPLC with MS is a powerful tool in biomedical analysis, since this detector provides high specifity and sensitivity. Today, the electrospray interface seems to be the most universal interface for LC–MS, providing the widest range of applications. Unfortunately, it is not possible to adopt every HPLC method for the use in LC–MS. There are several restrictions: only volatile buffers or volatile ion pairing reagents can be used, the flow-rate is limited. The more water or the less organic solvent is in the mobile phase the less flow is allowed. In our system, the optimal flow-rate was 75 μl/min. Using the described method we were able to separate α- and β-amanitin in urine extracts. The applied electrospray LC–MS technique allowed the ionization and detection of both amatoxins in the positive mode. In Fig. 4 the electrospray mass spectra, structures, empirical formulas and molecular masses of α- and β-amanitin are shown. Since both spectra contain the ions *m/z* 919 and 920 in different abundances, both ions were selected as diagnostic ions for detection of the amatoxins in the SIM mode (Fig. 5, bottom). Blank urine samples were analysed to show that there were no interferences (Fig. 5, top). The limit of detection for both toxins was 10 ng/ml of urine (*S/N*=3).

Symptoms of an intoxication with amanita mushrooms do not appear before a lag time of about 8 to 24 h. In our experience, urine concentrations of α- and β-amanitin at this time range between 50 to 500 ng/ml. Therefore, the detection of the described

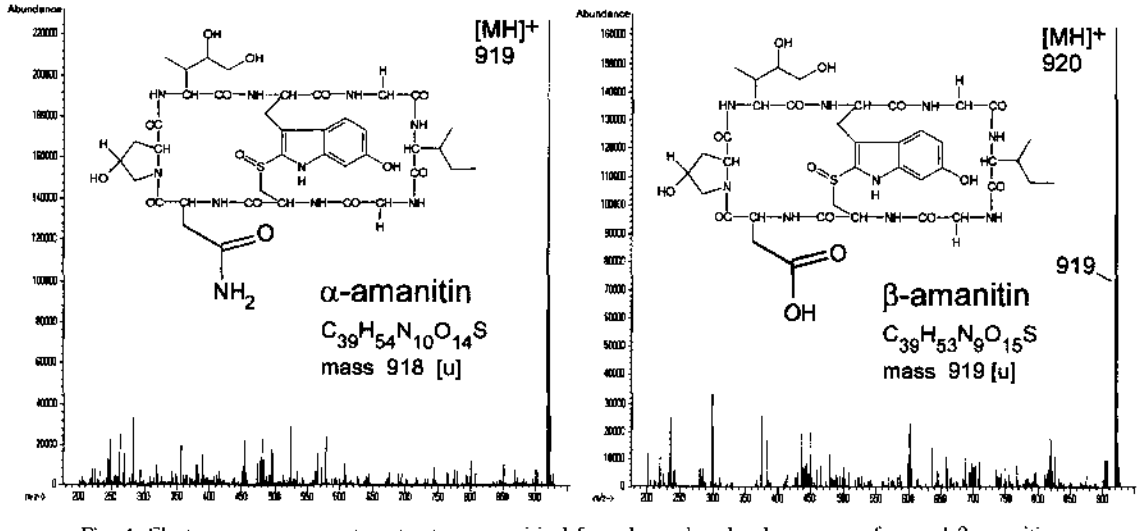

Fig. 4. Electrospray mass spectra, structures, empirical formulas and molecular masses of α- and β-amanitin.

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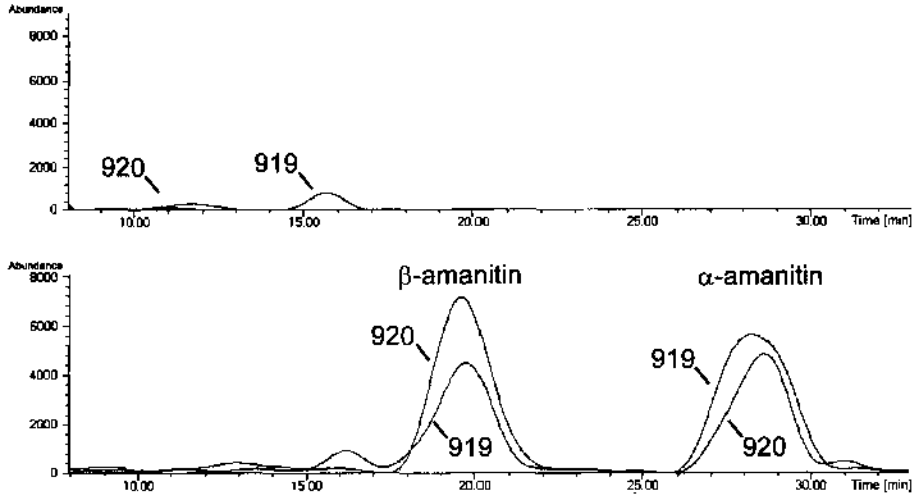

Fig. 5. Smoothed mass fragmentograms with the ions m/z 919 and 920 of extracts of a blank urine sample (top) and of a blank urine sample spiked with 50 ng/ml of α- and β-amanitin (bottom).

LC–MS procedure is suitable to diagnose an intoxication. In the meantime, we successfully applied our procedure in authentic clinical cases in which the RIA results could be confirmed by LC–MS.

4. Conclusions

The GC–MS procedure described here allows the precise and sensitive detection of clobenzorex and its metabolites in urine after therapeutic doses of clobenzorex within a systematic toxicological analysis. Other amphetamine derivatives as well as most of the toxicologically relevant drugs like barbiturates and other sedative–hypnotics, anticonvulsants, benzodiazepines, antidepressants, phenothiazine and butyrophenone neuroleptics, central stimulants, designer drugs, phencyclidine, opioids, non-opioid analgesics, antihistamines, antiparkinsonians, beta-blockers, antiarrhythmics, laxatives and their metabolites [3] could also be detected and differentiated within the same procedure by clicking the corresponding pull down menu (e.g. "amphetamine class") followed by library search of the spectra underlying the peaks.

The LC–MS procedure presented here allows the precise and sensitive detection of α- and β-amanitin in urine, thus allowing the specific diagnosis of an intoxication with amanita mushrooms.

These examples show that GC–MS is the method of choice for systematic toxicological analysis of GC volatile toxicants and that LC–MS is the method of choice for the detection of non-volatile toxicants.

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Drugs and dilated cardiomyopathies: a case/noncase study in the French PharmacoVigilance Database

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G.M. and S.F. performed the study in the FPVD. G.M., S.F., A.S., J-L.M. and M-C.P-P. analysed the data. J-L.M. wrote the manuscript. A.S., A.P., M.L-M. and M-C.P-P. revised the text.

Keywords
adverse drug reactions, anthracyclines, dilated cardiomyopathy, pharmacoepidemiology, pharmacovigilance

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Dilated cardiomyopathy is a frequent disease, responsible for 40–50% of cases of heart failure.
- Several aetiologies have been reported: idiopathic, familial/genetic, viral and/or immune but also toxic agents (alcohol, cobalt, carbon monoxide, lead, cocaine, mercury or drugs).
- Among drugs, anthracyclines are well known to induce such an adverse drug reaction.

WHAT THIS STUDY ADDS

- This study describes an association with already suspected drugs (anthracyclines, antiretrovirals).
- It also found a signal with other drugs (antipsychotics, lithium, antidepressants, retinoids).
- This pharmacovigilance signal should be confirmed by further prospective studies.

AIMS

To evaluate putative associations between drugs and dilated cardiomyopathy.

METHODS

We used the case/noncase method in the French PharmacoVigilance Database (FPVD). Cases were all the observations with dilated cardiomyopathy registered into the FPVD between 1 January 1990 and 30 June 2007. Noncases were all other reports other than those studied. Anthracyclines were used as positive controls. Data were expressed as reporting odds ratio (ROR) with their 95% confidence intervals.

RESULTS

Out of the 258 729 adverse drug reaction (ADR) reports recorded in the FPVD between 1 January 1990 and 30 June 2007, 47 (22 men, mean age 49 years) were defined as dilated cardiomyopathy. In these 47 patients, 67 drugs were 'suspect'. A significant ROR was found with cytotoxic (epirubicin, mitoxantrone, cyclophosphamide, gemcitabine, fluorouracil) and antiretroviral (lamividune, zidovudine, abacavir) but also with isotretinoin, prednisone, appetite suppressant (clobenzorex) and psychotropic [antipsychotic (clozapine, olanzapine), lithium, antidepressant (clomipramine, amitriptyline, fluvoxamine)] drugs.

CONCLUSIONS

The present study describes an association between some drugs and reports of dilated cardiomyopathies. This relationship involves not only some already suspected drugs (anthracyclines, antiretrovirals), but also other drugs (antipsychotics, lithium, antidepressants, retinoids) less known to induce such an ADR. Despite the mandatory limits of this kind of study (underreporting, confounding factors . . .), these data represent a pharmacovigilance signal and could contribute to establish further prospective studies in order to confirm such signals.

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Introduction

Dilated cardiomyopathy is defined by the presence of left ventricular systolic dysfunction in the absence of abnormal loading conditions (arterial hypertension, valve disease) or coronary artery disease sufficient to cause global systolic impairment [1]. It is a frequent disease, known to be responsible for 40–50% of cases of heart failure. Right ventricular dilation and dysfunction may be present but are not necessary for the diagnosis. Dilated cardiomyopathy is characterized by ventricular remodelling producing chamber dilation, with normal or decreased wall thickness and diminution in systolic function [2]. Congestive heart failure may or may not supervene. Impairment of systolic function can involve the left, right or both ventricles with an ejection fraction defined as <40% [3]. Presentation with disturbances of ventricular or atrial rhythm is common and death could occur at any stage. Its prevalence in the general population remains unknown, but at least 25% of patients in Western populations have evidence for familial disease with predominantly autosomal dominant inheritance [1]. Several aetiologies have been reported for dilated cardiomyopathies: idiopathic cardiomyopathy is a primary, global, myocardial disorder of unknown cause. Dilated cardiomyopathy could also be familial/genetic, viral and/or immune and related to some toxic agents [4, 5]. Besides alcohol, cobalt, carbon monoxide, lead, cocaine and mercury, exogenous agents potentially inducing dilated cardiomyopathy could be drugs: the main pharmacological class known to induce such a serious adverse drug reaction (ADR) is anthracyclines [5, 6]. Some cases reports or publications have also discussed the involvement of other drugs [5]. However, data remain conflicting.

Thus, the aim of the present study was to evaluate associations between drugs and reports of dilated cardiomyopathy, using the case/noncase method in the French PharmacoVigilance Database (FPVD).

Methods

Case/noncase method

The case/noncase approach measures disproportionality of combination between a drug and a particular ADR in a PharmacoVigilance Database [7–11]. Cases are reports corresponding to the ADR and noncases all other reports. The method allows comparison of drug exposure among cases and noncases and calculation of an ADR reporting odds ratio (ROR) with its 95% confidence interval (CI).

Source: the French PharmacoVigilance Database

The reporting of ADRs has been compulsory in France since 1984. According to the law, physicians must report 'serious' or 'unexpected' ADRs to their regional pharmacovigilance centre (31 in France). All suspected ADRs are registered in the FPVD [12]. For each report, information about patient (age, gender, medical history) and drug exposure (suspected and other associated nonsuspected drugs) are recorded in the FPVD. Causality assessment (imputation) is performed according to the French method used by all the Regional Centres of PharmacoVigilance [12]. A detailed summary of clinical description is added at the end of each pharmacovigilance case report. ADRs are coded according to ADR terminology of the World Health Organization (WHO-ART) [13]. 'Serious' ADRs were defined as reports leading to death, hospitalization (or prolongation of hospitalization), persistent or significant disability or incapacity, or being life threatening [14]. We investigated ADRs recorded in the FPVD between 1 January 1990 and 30 June 2007.

Selection of 'cases' and 'noncases'

As a first step, we identified all cases recorded during this period and including a code related to a cardiomyopathy (WHO-ART code 0425.001). All these reports were carefully read. Second, we included in the study only reports in accordance with the diagnosis of dilated cardiomyopathy according to the WHO/International Society and Federation Cardiology task force on the definition and classification of cardiomyopathies [4]. Analysis of each report was carefully performed by a specialist in clinical cardiology (G.M.). Only drugs for which two or more reports of dilated cardiomyopathy have been registered in the FPVD were investigated in the case/noncase analysis. When the reporting form was not sufficiently informative or when the characteristics of ADRs could not be completely defined, the report was not included in the analysis.

'Noncases' were defined as all other reports (i.e. all other reports of ADRs, not defined as dilated cardiomyopathy) registered between 1 January 1990 and 30 June 2007 in the FPVD. We included all reports (coded according to the International Classification of Diseases 9th edn) of whatever age or gender.

Drug exposition (coded according to Anatomical Therapeutic Chemical classification) was defined by the presence in the report of the drug of interest, whatever the level of causality assessment. In our analysis, we did not include drug doses, since it is not exhaustively recorded in reports.

Statistical analysis

Characteristics of patients, number and type of drugs were compared between reports defined as dilated cardiomyopathy (named 'cases') and all other reports in the database (named 'noncases'). We calculated an ADR ROR in order to compare the risk of exposure to the different classes of drugs in 'cases' and 'noncases'. RORs were given with their 95% CI as crude RORs [15].

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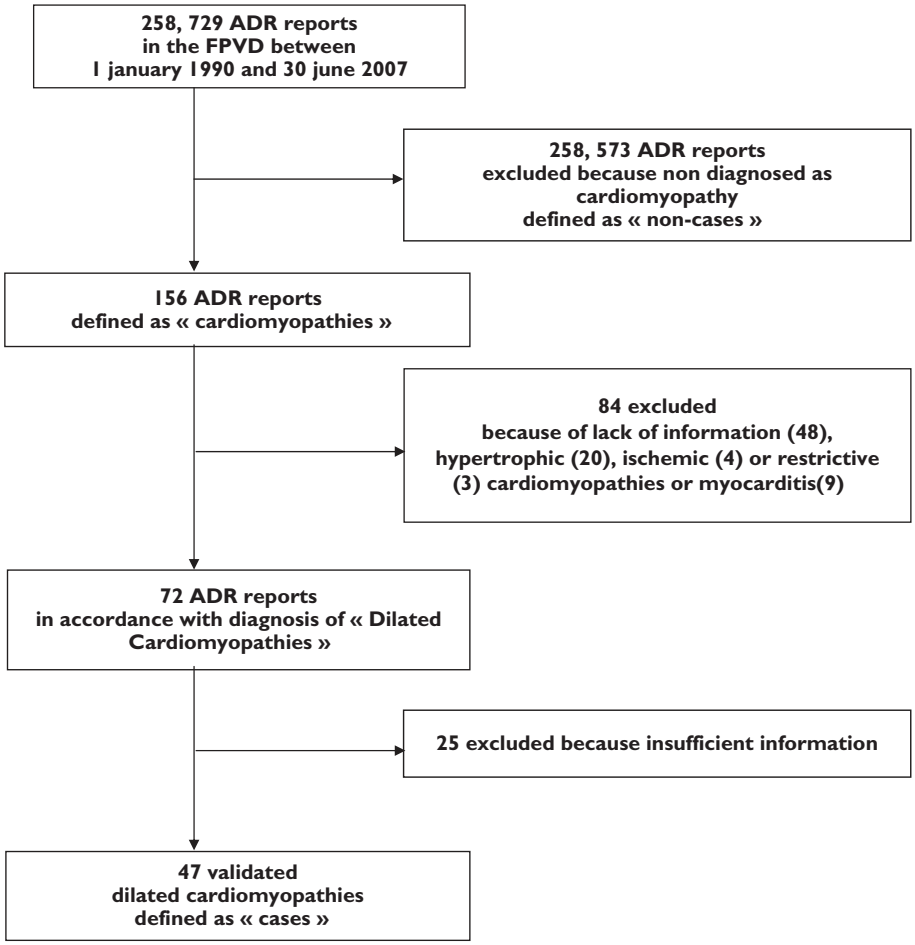
258, 729 ADR reports
in the FPVD between
1 january 1990 and 30 june 2007
258, 573 ADR reports
excluded because non diagnosed as
cardiomyopathy
defined as « non-cases »
156 ADR reports
defined as « cardiomyopathies »
84 excluded
because of lack of information (48),
hypertrophic (20), ischemic (4) or restrictive
(3) cardiomyopathies or myocarditis(9)
72 ADR reports
in accordance with diagnosis of « Dilated
Cardiomyopathies »
25 excluded because insufficient information
47 validated
dilated cardiomyopathies
defined as « cases »

Figure 1
Flow chart of the study. FPVD, French PharmacoVigilance Database; ADR, adverse drug reaction

Results

Characteristics of cases

Of the 258 729 reports recorded in the FPVD between 1 January 1990 and 30 June 2007, 47 were finally validated as dilated cardiomyopathy (Figure 1). Among them, 22 (46.8%) were observed in men. Mean age was 49.1 years (median 49.0 years).

Figure 2 shows that the maximal number of cases (n = 14) occurred between 40 and 49 years. No case was reported after 79 years.

Among the 47 case reports, 67 drugs were 'suspect'. In 36 observations, only one drug was defined as 'suspect'. In five cases, two different drugs were involved: epirubicin + fluorouracil (twofold), fluvoxamine + lithium (onefold), fluvoxamine + olanzapine (onefold) and zidovudine + lamivudine (onefold). Five other case reports involved three suspected drugs: zidovudine + abacavir (fourfold) and zidovudine + lamivudine + didanosine (onefold), whereas one notification involved four suspected drugs: didanosine + lamivudine + lopinavir + ritonavir.

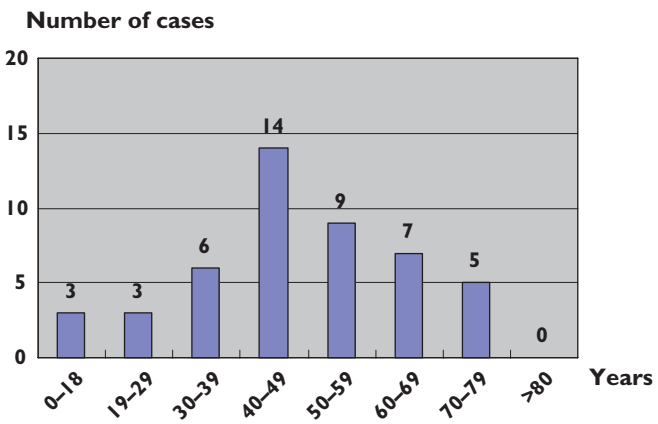

Figure 2
Repartition of the 47 cases of dilated cardiomyopathies according to age

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Table 1

Risk of exposure to drugs in cases (n = 47 with 67 suspect drugs) and noncases (n = 258 664) and occurrence of dilated cardiomyopathy in the French PharmacoVigilance Database (FPVD)

Pharmacological classes	Drugs	Number of dilated cardiomyopathies in the FPVD	Number of ADRs in the FPVD	ROR (95%CI)
Appetite suppressants	Clobenzorex	2	67	122.4 (29.3, 510,4)*
Antineoplastics				
Anthracyclines and derivatives	Epirubicin	4	256	65.1 (23.5, 180.2)*
Alkylating	Mitoxantrone	2	209	38.4 (9.3, 157.9)*
Antimetabolites	Cyclophosphamide	7	1126	26.8 (12.2, 58.8)*
	Gemcitabine	2	565	14.1 (3.4, 57.7)*
	Fluorouracil	4	1494	10.9 (3.9, 30.1)*
Antipsychotics	Clozapine	4	866	18.9 (6.9, 52.2)*
	Olanzapine	3	821	14.7 (4.6, 47.1)*
Mood stabilizers	Lithium carbonate	3	745	16.2 (5.1, 51.9)*
Antidepressants				
Imipraminic	Clomipramine	4	1418	11.5 (4.2, 31.7)*
	Amitriptyline	3	1129	10.7 (3.3, 34.1)*
Serotoninergic	Fluvoxamine	2	820	9.7 (2.3, 39.6)*
Retinoids	Isotretinoin	3	873	13.8 (4.3, 44.3)*
Glucocorticoids	Prednisone	3	1849	6.5 (2.0, 20.8)*
Antiretrovirals				
Nucleoside and nucleotide	Lamivudine	7	4436	6.7 (3.0, 14.6)*
reverse transcriptase inhibitors	Zidovudine	6	4344	5.7 (2.4, 13.3)*
	Abacavir	4	1919	8.5 (3.0, 23.4)*
	Didanosine	2	2113	3.7 (0.9, 15.2)

*P < 0.01, ROR, reporting odds ratio; 95% CI, 95% confidence interval. Only drugs for which two or more reports of dilated cardiomyopathy have been registered in the FPVD were included in the study.

Case/noncase comparison

The case/noncase comparison included the 47 patients ('cases') and the 258 729 reports ('noncases'), i.e. the 84 + 25 patients excluded for lack of information on other causes of cardiomyopathies were not included in the study. In these 47 patients, 67 drugs were defined as 'suspect' (i.e. possibly associated with the occurrence of ADR whatever the level of causality assessment). Table 1 indicates the values of ROR between occurrence of dilated cardiomyopathy and exposure to drugs in the FPVD.

Significant RORs were found for appetite suppressant (clobenzorex), cytotoxic (epirubicin, mitoxantrone, cyclophosphamide, gencitabine, fluorouracil), antipsychotic (clozapine, olanzapine), lithium, antidepressant (clomipramine, amitriptyline, fluvoxamine), isotretinoin, prednisone and antiretroviral (lamivudine, zidovudine, abacavir) drugs. In contrast, no significant association was found for didanosine.

Discussion

In order to better define involvement of drugs in dilated cardiomyopathy (a 'rare' ADR), the present study investigated putative associations between reports of dilated cardiomyopathy and exposure to drugs. We worked in the FPVD, a large PharmacoVigilance database (>350 000 case reports from 1985) allowing such investigations. Cases were carefully selected by a specialized clinical cardiologist in order to restrict our analysis to true validated dilated cardiomyopathies. Positive associations were found with some expected drugs (anorexic, antineoplasic and antiretroviral drugs) but also with other unexpected drugs used in psychiatric, dermatological or endocrinological diseases.

Our study suffers from some compulsory methodological drawbacks. The first problem is underreporting, a well-known phenomenon in pharmacovigilance surveys. Estimation could be biased according to several factors, one of the most important being the characteristic of 'seriousness' of the ADR [16]. In fact, it is known that a 'serious' and/or 'unexpected' ADR occurring with a new drug is more likely to be reported than another kind of ADR. Moreover, ascribing a cardiac event (like dilated cardiomyopathy) to a drug is not easy. However, it is important to underline that the work was performed in a context of signal detection: thus, we chose to be sensible rather than unable to identify a true association. In fact, the values of the RORs should be considered only as a signal and not as a true risk value.

The choice of the control group is another important limitation of case/noncase methodology. Results should be inferred only to patients who have suffered from an ADR. In fact, one of the limitations of the present study is that we did not account for some important factors such as smoking, alcohol, cardiac or familial antecedents (they were not always as fully informed in cases as well in noncases) . . . Alcohol is known to be an important factor in the pathophysiology of dilated cardiomyopathies [5]. Finally, the present study should be considered as exploratory.

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However, despite its mandatory limits, use of case/noncase methodology is now recognized as very useful in order to generate signals in PharmacoVigilance and drug safety research [17].

Taking into account these inherent limits, our study suggests that some cytotoxic and antiretroviral drugs, but also psychotropic, dermatological and endocrine drugs, could be associated with dilated cardiomyopathy. These findings may have three explanations: the underlying disease for which these drugs are used could be risk factor for dilated cardiomyopathy; the drug could be an innocent bystander; or the relation could be causal. In fact, with the notable exceptions of human immunodeficiency virus (HIV) infection (which could induce, at least partly, such a cardiac disease) and glucocorticoids, the two first explanations are unlikely due to the strong association found with most of the drugs. Moreover, careful revision of cases allows us to exclude other co-prescribed drugs in the pathophysiology of these dilated cardiomyopathies.

It is interesting to discuss the results from a pharmacodynamic point of view. First, our study confirms the risk of dilated cardiomyopathy with anthracyclines. This pharmacological class was selected as positive control in this study, as methodologically required [7–12]. This kind of cardiac ADR could occur in patients treated with anthracyclines but previously free of cardiac disorders [18–21]. In our study, association was found to be significant with epirubicin and mitoxantrone. However, no case was reported to the FPVD with other anthracycline antibiotics (such as daunorubicin or doxorubicin) known to induce such a serious ADR [22]. The reason remains unclear: underreporting, less marked use in France . . .

Other antineoplasic agents were found to be associated with dilated cardiomyopathy in our study: an alkylating agent (cyclophosphamide) and two antimetabolites (gemcitabine and fluorouracil). Dilated cardiomyopathies were not previously described with cyclophosphamide, a nitrogen mustard. In contrast, myocarditis induced by this alkylating agent is well documented and unknown myocarditis could lead to dilated cardiomyopathies. Incidence of myocarditis (after high doses of cyclophosphamide) has been estimated to 7–25% in adults and 5% in children [21]. In some cases, it could be related to total administered dose. The onset of myocarditis is acute and no late toxicity (>3 weeks) was reported. The precise mechanism is unknown and histopathological examination shows increased heart weight, thickening of the left ventricular wall and haemorrhagic myocardial necrosis [21]. In our study, no case was found with ifosfamide.

Among antimetabolites, an association between dilated cardiomyopathies and two pyrimidine analogues (fluorouracil and gemcitabine) was found in the present work. Fluorouracil-related cardiac events are complex and range from chest pain to massive myocardial infarction leading to cardiogenic shock and death. The topic is much discussed in the literature [20, 21]. Our work indicates that some cardiological complications of fluorouracil use could be dilated cardiomyopathy and also allows adding gemcitabine to the list of drugs potentially inducing this ADR.

It should be underlined that, in the present study, no case involved herceptin (a recombinant humanized anti-HER2 antibody), used in the treatment of breast cancer and known for its cardiac toxicity [20]. In fact, we found one cardiac case report inside the FPVD: however, the clinical description was not in accordance with the diagnosis of dilated cardiomyopathy and the observation was not included in the present study.

We should stress that the above-discussed antineoplasic drugs are frequently part of combination treatment regimens: thus, other associated antineoplasic drugs could also, at least in part, be responsible for the ADR (although they were not registered as 'suspect' in the case reports). However, in our study, ciclosporin was alone in two cases out of the seven and the two cases with gemcitabine only involved this drug.

One of the interesting results of our study was an association between some classes of psychotropic drugs (antipsychotics, lithium and antidepressants) and dilated cardiomyopathy. Among antipsychotics, a significant association was found with clozapine and olanzapine. In fact, myocarditis associated with clozapine has been known for several years. A recent paper reviewed 116 case reports submitted to the Australian Adverse Drug Reactions Unit between 1993 and 2003: calculated incidence was between 0.7 and 1.2% of treated patients. Patients were relatively young (mean age 30 years). More than 10% of the patients died [22]. Our results extend the conclusion of this large paper, indicating that clozapine could also induce dilated cardiomyopathy. The exact mechanism remains unknown, but this kind of ADR could be a class effect since it was also found with olanzapine, another antipsychotic drug structurally related to clozapine.

Among antidepressants, a significant association was found with both imipraminic (clomipramine, amitriptyline) and serotoninergic (fluvoxamine) antidepressants. In fact, some dilated cardiomyopathies with heart failure have already been reported with imipraminic antidepressants [20, 23–26], especially with high dosages [18]. Usually, symptoms disappeared after drug withdrawal. One publication described a positive rechallenge [26]. Pharmacological data suggest that the myocardial effects of antidepressants could be mediated by several mechanisms: atropinic and quinidine-like effects, interference with reuptake of adrenergic amines, alteration of membrane permeability or direct myocardial depression [20]. The observation of a significant association with fluvoxamine is more surprising, since this serotoninergic antidepressant is supposed to exert less direct effects on myocardial function than imipraminic compounds [27]. Thus, the results with antidepressants could represent a pharmacovigilance signal, which should be confirmed by other further studies.

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Another finding involves corticosteroids (and particularly prednisone). These drugs could exacerbate heart failure: this effect can be explained by their lateral mineralocorticoid properties, particularly after long-term use [18]. However, we cannot assume, from the results of the present study, that the described relationship with prednisone was causal: as discussed above, it could only be a indication of the underlying disease (infectious diseases in two cases and immunological disorder in one case) justifying prednisone prescription. In fact, the value of prednisone ROR was lower than that found with other drugs.

Antiretroviral agents are listed as one of the classical aetiologies of dilated cardiomyopathies [5]. In fact, this ADR has been reported with nucleoside and nucleotide reverse transcriptase inhibitors, including mainly zidovudine [27–30]. One case of dilated cardiomyopathy was previously published in a woman treated with zidovudine, lamivudine and nelfinavir: the dilated cardiomyopathy resolved after discontinuation of the three drugs and cardiac function remained normal with lamivudine, nelfinavir and abacavir, suggesting an important role for zidovudine [28]. Our study found such an association with zidovudine but also with lamivudine and abacavir (but not with didanosine). Dilated cardiomyopathy induced by antiretroviral agents is usually reversible, either spontaneously or with treatment. The mechanism of the dilated cardiomyopathy related to nucleoside and nucleotide reverse transcriptase inhibitors could involve drug-induced mitochondrial toxicity [30, 31]. However, one should stress that HIV infection alone is also able to induce such dilated cardiomyopathies [5]. Thus, the association found with antiretroviral drugs could be multifactorial, involving both the underlying disease and drugs.

One of the interests of this kind of research is to find new associations between occurrence of dilated cardiomyopathy and exposure to drugs. For example, we found only one case of dilated cardiomyopathy in the literature with clobenzorex [32] (an appetite-suppressant drug related to amphetamine drugs and known to induce primary pulmonary hypertension [33] but also cardiac valvulopathies [34]). The mechanism of this ADR remains unknown, although the description of such dilated cardiomyopathies with methamphetamine [35] could suggest involvement of catecholamine release and direct cardiac toxicity of catecholamine release.

Similarly, we were also unable to find, in literature, any report with isotretinoin (13- *cis*-retinoid acid), a vitamin A derivative which belongs to first-generation retinoids. Isotretinoin cardiac ADRs remain poorly described, even if one case of cardiomyopathy was previously found in a multicentre Phase II trial in association with interleukin (IL)-2 and IL-α in metastatic renal cell carcinoma [36]. Recently, two cases of isolated myocarditis were reported with tretinoin (all-*trans*-retinoid acid), another first-generation retinoid [37]. The proposed mechanism was suggested to be leucocyte infiltration and cytokine release causing endothelial damage and extravasations of acute promyelocytic leukaemia [37].

The present case/noncase study has also described an association with lithium. The effects of lithium on skeletal muscle are represented mainly by varying degrees of weakness and tremor. Acute or subacute painful proximal myopathy causing myalgia, cramps, myokymia or weakness has also been described. As far as we know, few case reports of cardiomyopathy associated with lithium have been published in the literature [38]. However, using the Bayesian confidence propagation network in the WHO database, Coulter *et al.* [39] found a significant association between lithium exposure and cardiomyopathy (but not myocarditis). The nature of the relationship (causal or incidental) remains a matter of discussion [38, 39].

In conclusion, the present study has described an association between some drugs and the reporting of dilated cardiomyopathies. This relationship involves, not only some already suspected drugs (anthracyclines, antiretrovirals), but also other drugs (antipsychotics, lithium, antidepressants, retinoids) less known to induce such an ADR. Despite the mandatory limits of this kind of study (underreporting, confounding factors . . .), these data represent a pharmacovigilance signal and could contribute to establishing further prospective studies in order to confirm such signals. From a practical point of view, the present study indicates that a systematic anamnesis about drugs should be involved in each aetiological research of dilated cardiomyopathy. Finally, these data also underline the interest of data mining in order to detect new signals of rare ADRs in a pharmacovigilance database.

Competing interests

None to declare.

The study was performed as a medical thesis by G.M.

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CLINICAL CASE

Amphetamine-related dilatated cardiomyopathy: a growing phenomenon. Case report

Cardiomiopatía dilatada por consumo de anfetaminas: un fenómeno creciente. Reporte de un caso

Carlos Alberto Solís-Olivares,* Héctor Alejandro Ramírez-García**

Key words:
Cardiomyopathy, amphetamines, methamphetamines, young, addiction, cardiotoxicity.

Palabras clave:
Cardiomiopatía, anfetaminas, metanfetaminas, jóvenes, adicción, cardiotoxicidad.

ABSTRACT

The consumption of amphetamines and its derivatives, either recreationally or for working reasons, is widespread, currently being the second most consumed drug worldwide. An often overlooked aspect of this drug has been its toxicity over the myocardium. In the following text we describe the case of a young male patient, without any relevant previous conditions, except alcohol ingestion in moderate quantity along with 3 years of using amphetamine-type stimulants and cocaine, whose main clinical manifestation was sudden onset dyspnea with minimal effort, which progressed to resting dyspnea after the ingestion of several tablets of clobenzorex, which was initially hospitalized with a diagnostic of pneumonia and finally diagnosed with a dilatated cardiomyopathy. Substance abuse should be a warning flag on patients presenting with dyspnea in order to think of a possible drug related cardiomyopathy, avoiding that way undue delays on appropriate diagnosis and treatment.

RESUMEN

El consumo de anfetaminas y sus derivados, ya sea recreativo o por razones de trabajo, es generalizado, siendo actualmente la segunda droga más consumida en el mundo. Un aspecto a menudo pasado por alto sobre esta droga, ha sido su toxicidad sobre el miocardio. En el siguiente texto, se describe el caso de un paciente varón joven, sin condiciones previas relevantes, excepto la ingesta de alcohol en cantidad moderada junto con tres años de uso de estimulantes de tipo anfetamínico y cocaína, cuya manifestación clínica principal fue disnea de inicio repentino con esfuerzo mínimo, que evolucionó a disnea de reposo tras la ingestión de varias tabletas de clobenzorex, que inicialmente fue hospitalizado con un diagnóstico de neumonía y finalmente diagnosticado con miocardiopatía dilatada. El abuso de sustancias debe ser un indicador de advertencia en los pacientes que presentan disnea para pensar en una posible cardiomiopatía relacionada con drogas, evitando así retrasos indebidos en el diagnóstico y tratamiento adecuados.

INTRODUCTION

Drug addiction is one of the main causes of burden on the global healthcare system, being in the top ten in the developed countries. The consumers of amphetamines or its derivatives represent around 1.1% of the global population.[1] The National Survey of Addictions (ENA) 2011, when compared to the ENA 2008, regarding the Mexican Northeast region, has shown a regional increase in the prevalence of both usages of any kind of drug (2.1% vs. 4.6%) and of illegal drugs (2.0% vs. 3.2%), which stands in sharp contrast to other areas of Mexico, in where no overall increase in drug consumption was observed, with the exception of the Western Region.[2] The last report of the Mexican national system of vigilance of addictions put the amphetamine type drugs as the third most important cause of interment in the national centers of treatment, only overcome by marijuana and alcohol. In the case of the northern region of the country this rises to the first place with 31.2% of all the interments.[3]

In regards to the use of amphetamines and its derivatives, generally consumed due to its weight-decreasing properties by the female population, there has been an increase in its use by the male population due to a work-

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related need, mainly by long-distance transport drivers, due to the need to remain awake for extremely long periods of time, which creates the conditions for an addiction.[4,5]

In the following case report we present a 22 years old patient with several risk factors associated with cardiomyopathy, the main one we purport to be a chronic use of amphetamines type stimulants, which presented to the emergency department with acute onset dyspnea. Amphetamine, along with its chemical analogue methamphetamine, are synthetic stimulants that act by increasing catecholamine concentrations in the central and peripheral nervous systems. Its cardiotoxic effect is thought to be due to exposure of the myocardium to excessive catecholamine levels, and amphetamine-inherent cardiotoxicity. The most severe acute cardiovascular complications include acute myocardial infarction, aortic dissection, and sudden cardiac death, while coronary artery disease, and cardiomyopathy, in both its dilatated and hypertrophic types, have been observed as chronic consequences of amphetamine use.[6]

Of interest in the following report was the three-day delay before a correct diagnosis could be established, despite the typical clinical presentation, due to the little-known association between chronic consumption of drugs and cardiomyopathy in the general medical community. This article aims to shed some light on its general pathophysiology and generate awareness about an ever-increasing problem at the global level, with an emphasis on the difference a prompt diagnosis and management can make in otherwise healthy young patients.

CASE REPORT

We present the case of a 22-year-old male patient, truck driver, with no relevant previous clinical history, with no reported congenital heart, or chronic-degenerative disease. The only thing of note is the occasional consumption of alcohol in moderate quantities, approximately 50 grams per week, along with the regular oral and nasal consumption of amphetamine-type stimulants and cocaine during the last 3 years in a non-specified quantity.

Two weeks before his hospitalization, during the day of 13/04/2016, he reported fits of dry cough, which became humid during the following week, along with the appearance of a sharp pain in the right hemithorax which was exacerbated by drawing breath. He didn't present fever, arthralgia, myalgia or shivering. The day of his internment he reported taking 12 tablets of amphetamines (clobenzorex 60 mg, for a total dose of 720 mg), presenting 8 hours later two episodes of vomit, along with a sudden increase in the intensity and duration of the fits of coughing and with sudden-onset dyspnea of minimal efforts, rapidly progressing to be symptomatic at rest. He also reported the presence of orthopnea, needing to be supported by 4 pillows to be comfortable. Due to all of this he presented to the urgency department of the hospital.

The patient presented with tachycardia, with a frequency of 145 bpm, polypneic with a respiratory frequency of 24 per minute, not capable of tolerating dorsal decubitus. No fever was reported. The blood oxygen saturation with no supplementary oxygen was reported at 84%, increasing to 92% with an oxygen mask with supplementary oxygen at 5 liters per min, keeping an steady blood pressure of 130/80 mmHg. The physical exploration revealed the presence of bilateral crackles at the pulmonary auscultation, with no apparent heart murmur. The chest radiography *(Figure 1)*, shows bilateral diffuse opacities along with cardiomegaly grade III.

The patient was admitted with an initial diagnosis of pneumonia, being placed under an antibiotic therapy with levofloxacin 750 mg IV q24 hrs, along with oseltamivir, nebulized salbutamol, and budesonide. During his third day of stay he presented a sudden severe worsening of his clinical condition, with increased polypnea, tachycardia, dizziness and chest pain, which required the initiation of non-invasive mechanical ventilation, along with a transfer to the intensive care unit (ICU). The electrocardiogram *(Figure 2)*, reported left ventricular hypertrophy as the most notable abnormality. A thorax CAT scan was performed *(Figure 3)*, which showed signs of acute pulmonary edema. Suspecting a cardiopathy, an echocardiogram was performed

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(Figure 4), which showed a depressed ejection fraction of 19%, along with severe generalized hypokinesis.

In the ICU treatment for heart failure was started, with levosimendan and diuretics, with marked clinical improvement during the following week, which allowed the transfer of the patient to the Internal Medicine Ward and his discharge the day after, with an appointment for outpatient vigilance. At his last day in the hospital his vital signs were stable, with a heartbeat of 87 bpm, 18 breaths per minute, peripheric oxygen saturation of 95%, blood pressure of 130/80 mmHg, with no fever or dyspnea and with no need of supplementary oxygen.

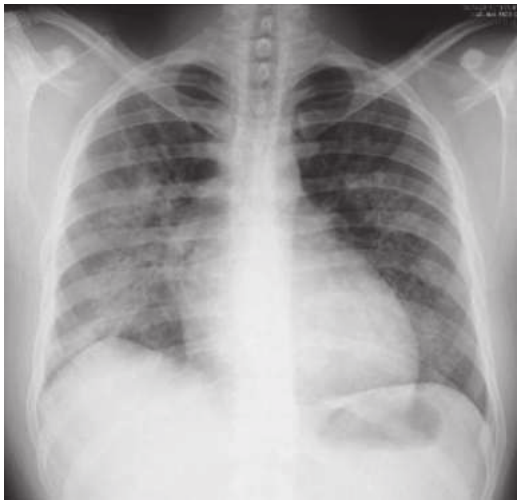

Figure 1. Simple PA thorax radiography, which shows cloudlike bilateral opacities along with disappearance of the right costodiaphragmatic recess, with a third degree cardiomegaly.

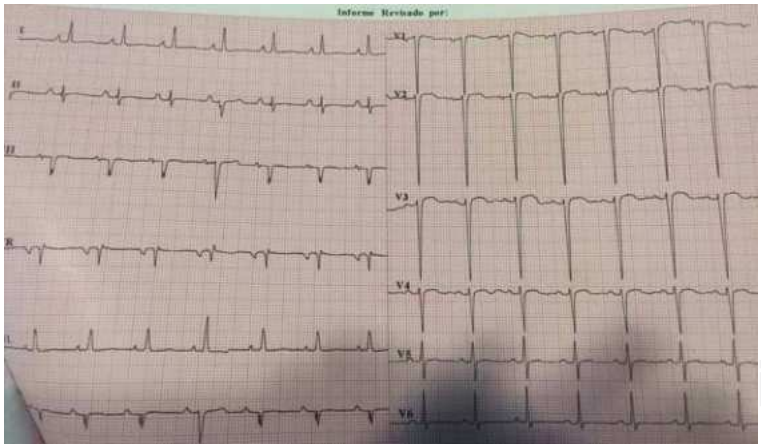

Figure 2. ECG which shows sinus rhythm, with a cardiac frequency of 93, PR 0.16 QRS 0.08, Axis at -30 degrees, without apparent signs of ischemia or necrosis, with presence of Left ventricular hypertrophy according to the Sokolow-Lyon and Cornell index.

DISCUSSION

Amphetamines are synthetic derivatives of phenethylamines, natural amines synthetized from phenylalanine. Adding a methyl group to amphetamines creates methamphetamines, which have a greater solubility in lipids and a greater capacity to cross the blood brain barrier. There exist also two isomeric forms of amphetamines, the dextro form being the most active at the central nervous system level, with 5 times the potency of its levo counterpart. Amphetamines are metabolized by the kidney, with a half-life of approximately 9 to 12 hours. Amphetamines don't have a sympathetic effect directly, instead they act through an extracytosolic increase of dopamine, norepinephrine, epinephrine, and serotonin in the synaptic space, through both mechanisms of increased release and inhibition of reuptake, which result in increased neuronal activity. Is has been postulated, thus, that this kind of substance harms the myocardium through a direct effect of excessive catecholamine stimulation, ischemia due to coronary vasospasm, mitochondrial

Figure 3. High resolution thorax computed axial tomography, in which it can be seen the presence of multiple hyperdense zones in the pulmonary parenchyma, with an increase in liquid in the peribronchial spaces, indicative of acute pulmonary edema.

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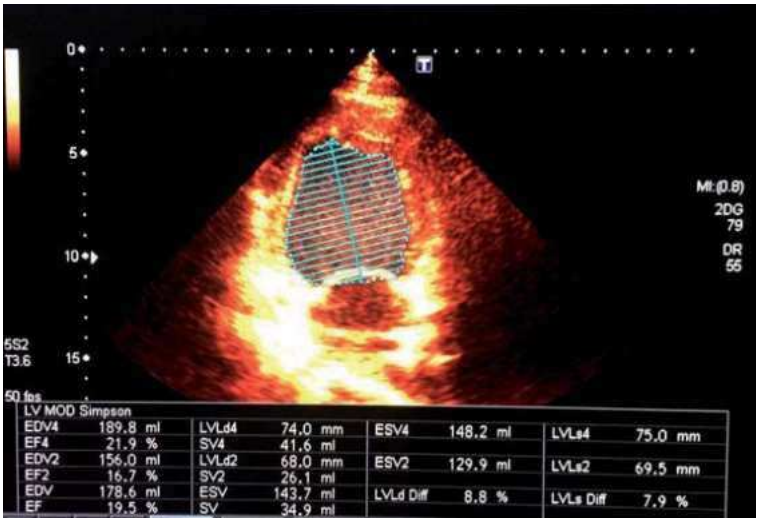

Figure 4. Transthoracic echocardiography, with measurement of the ejection fraction (EF) through Simpson's method, which shows EF of 19%.

damage and an increase in the presence of free oxygen radicals.[7] In contrast to this, cocaine inhibits directly the reuptake of norepinephrine in the synaptic space of sympathetic neurons, favoring the occurrence of coronary vasospasm, coronary thrombosis, direct toxic effect due to the excessive sympathetic stimulation and an absolute increase in the consumption of oxygen by the myocardium due to important tachycardia and an increase in the blood pressure levels.[8]

In the case of our patient, the only relevant clinical history for the development of a cardiopathy, with a severely depressed systolic dysfunction, was the start of consumption of both cocaine and amphetamines three years before the start of the symptoms. The initial confusion regarding the diagnosis is remarkable, as it was being treated as an infectious pulmonary pathology. This could be explained by the age of the patient, alongside the absence of traditional risk factors associated with cardiovascular disease with a decrease of the ventricular function, with it being an obscure diagnosis, even if multiple cases of ventricular failure related to drug consumption have been reported since at least the 80's.[9-11]

Alcohol consumption has been related to dilated cardiomyopathy,[12,13] however in the current case the quantity ingested, less than 40 grams per day, isn't considered enough to provoke acute damage to the myocardium. The use of cocaine has also been associated to dilated myocardiopathy in young patients, generally in their twenties to thirties, whose common initial presentation is acute chest pain and in some case signs of heart failure, with an abnormal echocardiogram. However, in the current literature it is described as uncommon the initial finding of a dilated cardiomyopathy in relation to the consumption of cocaine, being far more common the presence of hypertrophic cardiomyopathy, alongside changes in the EKG which show areas of both ischemia and scar tissue.[14] Due to this reasons we consider far more probable than the main etiological culprit of the dilated cardiomyopathy found in the patient is the consumption of amphetamines, with a possible synergistic effect with cocaine.[15] However, to date there haven't been any appropriate studies which show the impact of the concurrent use of multiple sympathetic illegal drugs on the myocardium and how it compares to single-use cardiomyopathy.[7]

The clinical effects of amphetamine type stimulants include an increase in energy, euphoria, energy and a reduction in appetite,[16] mainly acting through mesolimbic and mesocortical neuronal dopaminergic pathways.[7] Amphetamines and methamphetamines, besides being much cheaper than cocaine, also have a greater potency and a longer lasting effect, which has greatly contributed to the increase in national and international use during the last years, being right now the second most consumed illegal drug at the global level.[1] Another thing to bear in mind when treating this kind of patients is the possibility of a reversal of the cardiomyopathy if the patient ceases consumption of the offending drug, something of great relevance for the prognosis of this type of cases.

Amphetamines and methamphetamines are drugs that have become extremely popular at the global level, mainly in developing and newly industrialized countries. It is necessary to give a greater importance to the risk factor of illicit drug consumption in a patient's clinical history, mainly in the young with clinical signs of heart failure and dyspnea. Outreach and educational programs have to be established to give the necessary information to non-cardiologists, for even though there haven't yet been enough

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studies to obtain precise data on the quantity and time involved in the development of myocardiopathy related to the usage of amphetamines and methamphetamines, it is important to take it into account in the differential diagnosis, in order to guide our treatment, and give accurate information to the general population regarding the risk of consumption of this toxic substances and its potential reversibility if they cease consuming it.

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Diffuse Alopecia Areata Associated with Weight-Loss Pills

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ABSTRACT

Alopecia associated with ingestion of amphetamines has been reported occasionally. These drugs may act as a triggering factor to develop diffuse alopecia. We present a case of alopecia areata possibly linked to the ingestion of amphetamines. This case may provide evidence that amphetamines can also be a cause of alopecia areata in susceptible individuals. We intend to awaken an interest in the medication history of patients presenting with sudden diffuse alopecia areata.

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Key words: Alopecia, alopecia areata, amphetamines, medication-induced alopecia, weight loss

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INTRODUCTION

Alopecia related to the ingestion of amphetamines has been reported as early as 1965 with amphetamines or amphetamine-like drugs.[1] Some authors even suggest that the sole discontinuation of the drug can lead to regrowth of the hair.[2] There is little information linking the ingestion of these drugs as a triggering factor to develop alopecia. We present a case of alopecia areata possibly linked to the ingestion of amphetamines.

Case Report

A 50-year-old Hispanic female presented to the clinic with a 15-day history of diffuse alopecia. Her medical history was relevant to obesity for which she had been taking clobenzorex in the last 5 months (Itravil AP, 60 mg a day). The rest of her medical record was unremarkable. Laboratory workup and a biopsy were required. Her diagnosis was compatible with alopecia areata. Initial levels of Vitamin D and ferritin were 8 ng/mL and 25 ng/mL, respectively. She was then treated with oral mini-pulses of dexamethasone and azathioprine 100 mg as a steroid-sparing agent. Improvement was observed within a month of starting therapy. According to the Naranjo algorithm, our patient suffered a probable adverse drug reaction (5 points) to clobenzorex [Figure 1].

Amphetamines or amphetamine-like drugs have been prescribed for obesity, narcolepsy, as well as for pediatric disorders such as Attention Deficit/Hyperactivity Disorder (ADHD). The earliest report of alopecia secondary to the use of amphetamines was described as "diffuse thinning of hair on the vertex" in 1988 by Voron.[1] Another study regarding amphetamines and obesity with 2370 subjects reported nonclassified alopecia (1.7%) as a side effect.[3] Testing of lisdexamfetamine in adults mentioned an unclassified alopecia in one subject.[4] This drug was also responsible for a generalized alopecia in a 5-year-old girl treated for ADHD 5 days after starting treatment.

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Figure 1: (a) Clinical image of the patient at presentation; (b) clinical image of the patient at 6 months of follow-up; (c) trichoscopy (×20) showing black dots, broken hairs. (d) Trichoscopy with yellow dots, Pohl–Pinkus constriction, and vellus hairs (×20); (e) follow-up trichoscopy (×20) with full regrowth at 6 months

Her hair loss ceased 2 days after stopping medication.[5] Dextroamphetamine ingestion was associated with a sudden rapid loss of almost all body hair in a 57-year-old female with a long history of narcolepsy.[1] These reports may suggest a possible relation of amphetamine use with the development of alopecia. Some authors have stated that there is no causal relationship between the use of psychotropic medications and alopecia, because also rapid weight loss can contribute to it.[2]

CONCLUSION

The occurrence of this alopecia areata associated with the intake of clobenzorex (amphetamine) provides more evidence on the possible causality of ingestion of amphetamines and alopecia. This case intends to awaken an interest in the medication history of patients presenting with sudden diffuse alopecia areata that could possibly be associated with the intake of weight-losing medication, specifically amphetamines.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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shown to occur in TPN-dependent patients, due to lack of enteral intake stimulating bile flow and hepatocyte phytosterol accumulation interfering with bile acid secretion in patients utilizing soybean-oil lipid emulsions. Steatosis has been linked to TPN-induced depletion of carnitine and choline, which results in disrupted hepatocyte lipid transport and storage. Liver histology reveals cholestasis, portal inflammation, macrosteatosis, and ductular proliferation. TPN formulas with fish-oil and olive-oil lipid emulsions are emerging as less hepatotoxic. Ursodiol may reduce cholestasis and liver injury. Death is often due to liver disease, sepsis, and primary disease.

S3248

A Case of Drug-Induced Liver Injury Secondary to Clobenzorex Use

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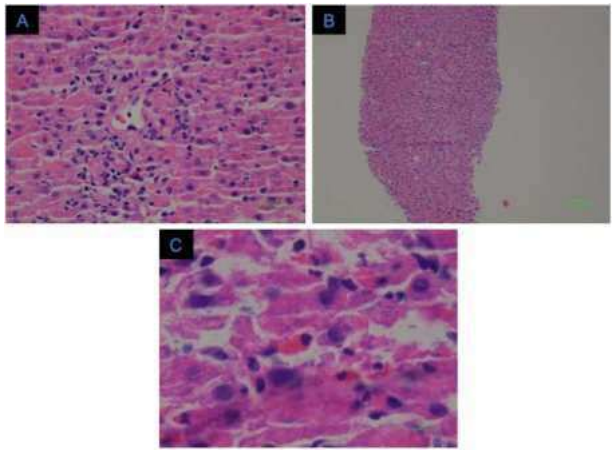
Introduction: Drug-induced liver injury (DILI) is a cause of both acute and chronic liver disease and is often a challenging condition for clinicians. It is a diagnosis of exclusion that requires a detailed history and workup to ensure other potential causes have been ruled out before affirming the diagnosis. There are numerous offending agents with varied presentations, often idiosyncratic in nature, with the additional challenge being a lack of specific testing or biomarkers to aid diagnosis.
Case Description/Methods: We present a case of a 25-year-old male who immigrated from Mexico 4 months ago, with no reported past medical history, who presented to the emergency department for a two-day history of postprandial non-radiating right upper quadrant and epigastric pain. Associated symptoms include subjective fevers, chills, decreased appetite, nausea, one episode of blood-tinged emesis, night sweats and dyspnea. The patient worked as a driver in Mexico and was taking two different formulations of 30mg Clobenzorex, an amphetamine pro-drug, daily for two years to help him stay awake for work. These medications were available over the counter. He had not taken it since he moved to the US. He denies the use of illicit drugs, contact with any inmates or Tuberculosis patients. At the time of his presentation, he works in a wooden floor-making company. Labs revealed a markedly elevated total bilirubin of 4.4 mg/dL, INR 1.1, AST 1325 IU/L, ALT 3151 IU/L, LDH 745 IU/L and a mildly elevated ALP 220 IU/L. Acute hepatitis and autoimmune panels were not reactive. Serum acetaminophen level was also within normal limits. Ultrasound of the abdomen and MRCP noted gallbladder wall thickening but no biliary dilatation or pathology. The patient subsequently underwent a core-needle liver biopsy that displayed lobular infiltrates of inflammatory cells of predominantly lymphocytes (Figure 1). Given the absence of positive viral serology, a diagnosis of drug-induced hepatitis was concluded.
Discussion: This case report documents a unique instance of drug-induced liver injury secondary to Clobenzorex use. This case highlights the importance of a detailed social history and should encourage clinicians not to neglect to ask about over-the-counter medications to help elucidate potential causes of liver injury. This is an important consideration, especially in the subset of patients from countries outside the United States who have access to a variety of OTC medications that are potentially hepatotoxic.



[3248] **Figure 1.** Core needle liver biopsy showing lobular infiltrates of inflammatory cells consisting of predominantly lymphocytes.

S3249

A Deadly Case of *Vibrio vulnificus* Bacteremia After Shellfish Consumption in a Patient With Cirrhosis

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Introduction: Infection with *Vibrio vulnificus* is the leading cause of death related to seafood consumption in the United States. The consumption of raw or undercooked seafood can cause bacteremia and sepsis with fatality rates >50%. Approximately 80% of patients who die from *V. vulnificus* infections have underlying liver disease.[1]
Case Description/Methods: The patient is a 51-year-old female with a history of decompensated alcoholic cirrhosis complicated by ascites and esophageal varices with a baseline MELD score of 17. She presented with one day of altered mental status, increased abdominal distension and lower extremity edema, skin bruising, and fever. On hospital presentation, she was afebrile, tachycardic, and normotensive. Labs revealed a Na 122 mmol/L, Cr 1.5 mg/dL, WBC 9,000 /mL, Total Bilirubin 6.8 mg/dL, INR 13, pH 7.17, lactate 13 mmol/L. Shortly after arrival, the patient had a cardiac arrest and return of spontaneous circulation was achieved after 1 round of chest compressions. She was admitted to the ICU on three vasopressors, bicarbonate, pantoprazole, and octreotide infusions, and broad-spectrum antibiotics with vancomycin and piperacillin/tazobactam. After two days she was transferred to our hospital where her exam revealed hemorrhagic bullae over abdomen and extremities (Figure 1). Her admission blood cultures eventually grew *V. vulnificus*. Upon questioning, the patient had eaten oysters and sushi three days prior to admission. Antibiotics were transitioned to doxycycline and cefepime though the patient had progressive shock and died four days after admission.
Discussion: This case demonstrates the severity and rapid progression of *V. vulnificus* infection in patients with underlying liver disease. The prognosis is linked to time to treatment. Studies have shown that patients who have treatment delayed for 72 hours have a mortality of 100%.[2] *V. vulnificus* is not covered by typical broad-spectrum antibiotics and delay in onset of treatment by enough time for a blood culture to speciate dramatically increases mortality. *V. vulnificus* is important to keep on the differential in any patient with gram-negative sepsis, skin wounds, and a history of liver disease. Questions about raw seafood and swimming in sea water should be routinely asked to guide clinicians to an early diagnosis. This case demonstrates a classic presentation of an uncommon infection and suggests that the clinician should consider empiric treatment for a *V. vulnificus* if there is a high index of suspicion.

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(+)-Clobenzorex Pharmacokinetic Profile

Chemical Properties

IUPAC Name: (2*S*)-*N*-[(2-Chlorophenyl)methyl]-1-phenylpropan-2-amine
CAS Name: (*S*)-*N*-[(2-Chlorophenyl)methyl]-α-methylbenzeneethanamine
INN: Clobenzorex
Other Name: (+)-*N*-(2-Chlorobenzyl)amphetamine
Trade Names: Itravil, Asenlix, Rexigen, Finedal, Dinintel, Obeclox, and others
Molecular Formula: $C_{16}H_{18}ClN$
Molecular Weight: 259.78 Da
Percent Composition: C 73.98%, H 6.98%, Cl 13.65%, N 5.39%

Physical Properties

pKa: 8.8–10.4
Melting Point: 455.15–456.15 K
Partition Coefficient: 4.27
Intrinsic Solubility: -5.39 mol/L
Density: 1.1±0.1 g/cm³

ADME & ADMET

Time of Onset: 1–1.5 h
Time of Peak: 3–4 h
Time of Offset: 7–15 h
Elimination Half-Life: 11.1±1.4 h
Protein Binding: 15–30%
Primary Metabolites: 4HC (10.1–40.4%), DAM (5.9–15.4%), unchanged CLX (1–2%), and *N*-(2-chloro-4-HO-benzyl)amphetamine (N.D.)
Secondary Metabolites: 4-HO-Amphetamine, norephedrine, phenylacetone, 3,4-DiHO-clobenzorex, *N*,4-DiHO-clobenzorex, and *N*-(2-chloro-4-HO-benzyl)-4-HO-amphetamine
Teritary Metabolites: 4-HO-Norephedrine, 4-HO-phenylacetone, benzoic acid, and *N*-(2-chloro-4-HO-benzyl)-4-HO-amphetamine
Quaternary Metabolite: Hippuric acid
$ED_{50} = 1.069$ mg/kg
$LD_{50} = 8.343$ mg/kg
$95\%CL = 0.648–1.750$ mg/kg

Pharmacokinetic Parameters

Metric data calculated following the oral administration of a single 30 mg amount by the average weight for an adult male (70 kg)

CLX:	DAM:	4HC:
$A = 30$ mg	$A = 1.77–4.62$ mg	N.D.
$D = 0.43$ mg/kg	$D = 0.025–0.066$ mg/kg	N.D.
$C_{max} = 0.01–0.029$ mg/L	$C_{max} = 0.715–2.474$ mg/L	$C_{max} = 5.705–88.41$ mg/L
$t_{max} = 1.5±0.2$ h	$t_{max} = 3.3±0.6\ h$ h	N.D.
$t_{1/2} = 3.1±0.3$ h	$t_{1/2} = 8±1.1$ h	N.D.
$k_e = 0.2–0.25$ h^{-1}	$k_e = 0.076–0.1$ h^{-1}	N.D.
$V_D = 15–43$ L	$V_D = 0.027–0.035$ L	N.D.
$CL = 3–10.75$ L/hr	$CL = 0.0021–0.0035$ L/hr	N.D.
$LoD = 0.001$ mg/L	$LoD = 0.005$ mg/L	$LoD = 0.001$ mg/L
$LoQ = 0.001$ mg/L	$LoQ = 0.005$ mg/L	$LoQ = 0.025$ mg/L
$Dt_0 = 1.5–5$ h	$Dt_0 = 1–5$ h	$Dt_0 = 1–5$ h
$Dt_{last} = 5–29$ h	$Dt_{last} = 77.5–116$ h	$Dt_{last} = 45.5–91.5$ h

!~ Metabolic Pathways !~

CYP2D6:	CYP3A4:	CYP2C19:	CYP1A2:	CYP2B6:
$K_i = 0.1549$ or 0.1738 μM	$K_i = 0.2754$ or 0.3467 μM	$K_i = 1.3803$ μM	$K_i = 15.4871$ μM	N.D.

norephedrine; *para*-hydroxylation of norephedrine into 4-HO-norephedrine	Role: *N*-Dealkylation of CLX into DAM	Roles: *para*-Hydroxylation of CLX into 4HC and/or *N*-(2-chloro-4-HO-benzyl)amphetamine; *para*-hydroxylation of 4HC into 3,4-DiHO-clobenzorex and/or *N*,4-DiHO-clobenzorex; *para*-hydroxylation of *N*-(2-chloro-4-HO-benzyl)amphetamine into *N*-(2-chloro-4-HO-benzyl)-4-HO-amphetamine	Roles: *para*-Hydroxylation of CLX into 4HC and/or *N*-(2-chloro-4-HO-benzyl)amphetamine; *para*-hydroxylation of 4HC into one or both of its conjugates; *para*-hydroxylation of *N*-(2-chloro-4-HO-benzyl)amphetamine into its conjugate	Roles: *N*-Dealkylation of CLX into DAM; *para*-hydroxylation of CLX into 4HC and/or *N*-(2-chloro-4-HO-benzyl)amphetamine; *para*-hydroxylation of 4HC into one or both of its conjugates; *para*-hydroxylation of *N*-(2-chloro-4-HO-benzyl)amphetamine into its conjugate

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List of Abbreviations, Symbols, and Terms

- CLX: (+)-Clobenzorex or clobenzorex
- DAM: (+)-Amphetamine or d-amphetamine
- 4HC: 4-HO-clobenzorex
- N.D.: No data available
- S.D.: Single dose, mg
- A: Amount, mg
- D: Dose, mg/kg
- C_{max}: Peak plasma concentration, mg/L
- t_{max}: Time to C_{max}, h
- $t_{1/2}$: Elimination half-life, h
- V_D: Apparent volume of distribution, L
- k_e: Elimination rate constant, h^{-1}
- CL: Clearance, L/hr
- LoD: Limit of detection, mg/L
- LoQ: Limit of quantitation, mg/L
- Dt_0: Intial detection time, h
- Dt_{last}: Last detection time, h
- $95\%CL$: Dose to reach 95% clearance, mg/kg
- ED_{50}: Median effective dose, mg/kg
- LD_{50}: Median lethal dose, mg/kg
- K_i: Inhibitor constant, μM

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INVOLVEMENT OF HUMAN HEPATIC CYTOCHROME P450 ISOENZYMES IN THE METABOLISM OF THE AMPHETAMINE PRECURSOR DRUGS CLOBENZOREX AND MEFENOREX

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Aims: There is still no general consensus on which cytochrome P450 (CYP) isoenzymes are involved in the metabolism of amphetamine precursor drugs. Previous Studies on Wistar and Dark Agouti rats indicated that the polymorphically expressed human isoenzyme CYP2D6 might play a major role in metabolism of such drugs. The aim of our present study was to confirm this assumption by identifying the human hepatic CYP isoenzymes involved in N-dealkylation as well as in hydroxylation of the amphetamine precursors clobenzorex and fenproporex.

Methods: Activity screenings with nine individual cDNA expressed CYPs for general involvement were performed. Kinetic profiles were established using recombinant baculovirus infected insect cell microsomes and pooled human liver microsomes as enzyme sources. Experiments with chemical inhibitors completed the studies. Metabolites were quantified by NICI-GC-MS (HP5-MS column) after aqueous derivatization of the amine group with S-(-)-heptafluorobtuyrylprolyl chloride, liquid-liquid extraction and subsequent ethylation or heptafluorobutyrylation of the hydroxy groups.

Results: Besides CYP3A4, the most abundant human CYP isoform, CYP2B6 was involved in the N-dealkylation of clobenzorex to amphetamine. N-dealkylation of fenproporex was catalyzed by CYP3A4, CYP1A2, CYP2B6 and by CYP2D6. Hydroxylation to different hydroxy metabolites of clobenzorex was catalyzed by CYP1A2, CYP2B6 and also by CYP2C19. Hydroxylation to hydroxy fenproporex was catalyzed by CYP1A2, CYP2C19, CYP3A4 and also by CYP2D6. The kinetic constants, K_m and V_{max}, were determined for both enzyme sources.

Conclusions: As CYP3A4 is responsible for N dealkylation of both precursors, interactions with inhibitors or inducers of this isoenzyme are possible. Since CYP3A4 is the most abundant CYP isoenzyme in human liver, a major influence of polymorphisms and/or induction of other isoforms involved seems not likely. Concerning hydroxylation, different pharmacokinetic behaviours might be possible in vivo, because CYP2C19 and CYP2B6 are expressed polymorphically in humans and CYP1A2 is known to be inducible e.g. by smoking. However, additional involvement of CYP3A4 in fenproporex hydroxylation reduces the probability of different pharmacokinetic behaviour for this precursor caused e.g. by CYP2D6 polymorphism. The results of the study raise the question, why the Dark Agouti rat model overestimated the role of polymorphic CYP2D isoenzymes in amphetamine precursor metabolism.

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RECENT ADVANCES IN DOPING ANALYSIS (5)

W. Schänzer
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A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1998

A. TSOUTSOULOVA-DRAGANOVA, N. HALATCHEVA, D. KAROVA:
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Metabolism of Benzphetamine and Clobenzorex in human urine

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ABSTRACT

The metabolism of the stimulants Benzphetamine and Clobenzorex in the human urine after a single oral dose are investigated. Several new hydroxymetabolites of Benzphetamine and Clobenzorex as well as the well-known metabolites - amphetamine, methamphetamine and desmethylbenzphetamine are found and identified in the collected urines upto 48th hour after administration. Conjugated urinary metabolites are determined after hydrolysis. Their structures are identified by gas chromatography/mass spectrometry using the different derivatisation procedures - methylation, silyllation, selective derivatization, cyclization with methaneboronic acid. The fragmentation of some type of different derivatives is discussed and the probable metabolism scheme of Benzphetamine and Clobenzorex is proposed.

Key words: Stimulants, Benzphetamine, Clobenzorex, Doping analysis, Urine, Metabolism, Gaschromatography/Electron impact Mass spectrometry.

INTRODUCTION

Many drugs, enclosed in the list of banned stimulants for athletes produce in human urine amphetamine as its primary metabolite and in minor extent of p-hydroxy-amphetamine. At the time, the literature data show that the applied substance could be change depending on the temperature and the duration of the sample storage [1]. Therefore the detection of amphetamine in the screening procedure of free stimulants could be submit to the analyst the

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question: **What is the pattern substance?** For this reason the study gives attention to the detection of other metabolites that could be prove the origin of the administrated drug.

Benzphetamine, (S)-N, α - Dimethyl-N-(phenylmethyl) benzeneethanamine and Clobenzorex, (+)-N-[(2-Chlorophenyl)methyl]-α-methylbenzeneethanamine have similar chemical structures and an anorexic therapeutic effect.

The aim of this work is to describe the obtained results on the metabolism of Benzphetamine and Clobenzorex in collected urine samples up to 48 hours after oral administration of one therapeutic dose. The investigation was performed with extracts of urine samples obtained without hydrolysis and after enzyme hydrolysis by Helix Pomatia. The chemical conditions of screening procedures for free and conjugated stimulants and narcotics were used. The analytical determinations are performed by gas chromatography/electron impact-mass spectrometry (GC/EI-MS). In order of receiving a better idea about the structures of the detected metabolites were used the different derivatization procedures as methylation, silyllation, selective derivatization and cyclization with methaneboronic acid.

EXPERIMENTAL

Chemical and reagents

All reagents were of analytical grade. Double distilled diethylether stored in glass bottle in a refrigerator was used. Anhydrous sodium sulphate was dried at 300°C for 12 hours.

Stock standard solutions of Diphenylamine (DPA), Bamethane (ISTD), Amphetamine and methamphetamine were prepared in methanol. These solutions were stored at -20°C.

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), N-Methyl-bis-trifluoroacetamide (MBTFA) were obtained from Machery-Nagel and methyliodide and methaneboronic acid (MBA) - from Fluka.

β-Glucuronidase/arylsulphatase from Helix Pomatia was purchased from Sigma.

Excretion study

Two benzphetamine (25 mg) tablets Inapetyl®and a clobenzorex (30 mg) tablet Dinintel® were taken orally by healthy volunteers. The urine samples were collected up to 48 h and stored at 4°C until analysing.

Sample preparation

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For free stimulants: 5 ml of urine were added to 50 µl of internal standard working solution to obtain a concentration of 0.5 µg/ml Diphenylamine (DPA) and alkalised by 0.5 ml of 20% NaOH (pH 14). Two ml of distilled diethylether and 3 g of anhydrous sodium sulphate were added to the solution. The sample was roller mixed for 30 min. Then, it was centrifuged at 2500 rpm for 5 min. The organic layer was transferred to another tube and concentrated to 1 ml by evaporation under the stream of nitrogen. 2 µl were injected into the GC.

For conjugated stimulants: Urine sample (5 ml) was added to 20 µl of internal standard working solution to obtain a concentration of 0.5 µg/ml bamethane (ISTD). Then, 1 ml of 1N acetate buffer (pH 5.2) and 50 µl of ß-glucuronidase/arylsulphatase from Helix Pomatia were added. The sample was vortex mixed, heated to 55°C for 3 h or for one night at 37°C in a block-heater. The sample was cooled to room temperature and extracted by 5 ml of distilled diethylether in a shaker for 20 min. Then, it was centrifuged at 2500 rpm for 5 min.
The organic layer was discarded. The aqueous phase pH was adjusted to 9.6 ± 0.1 with $NaHCO_3/K_2CO_3$ (1:2 w/w) and were added 0.5 ml tert-butanol, 5 ml of distilled diethylether and 3 g of anhydrous sodium sulphate. The mixture was extracted by shaking for 20 min, then centrifuged at 2500 rpm for 5 min. The organic layer was transferred in a derivatisation vial and evaporated to dryness under stream of nitrogen in a 50°C water bath. The sample was kept in a desiccator over P_2O_5/KOH for 30 min.

Derivatisation

Preparation of O-TMS, N-TMS derivatives: To the dried residue was added 50 µl MSTFA and heated for 20 min at 80°C. After cooling to room temperature 1 µl of the sample was submitted to GC/MSD.

Preparation of O-TMS, N-TFA derivatives: To the dried residue was added 50 µl MSTFA and heated for 5 min at 80°C. After cooling to room temperature 15 µl of MBTFA was added and heated again for 10 min at 80°C. The sample was cooled and 1 µl was submitted to GC/MSD.

Preparation of n-methylboronates: To the dried residue was added 100 µl solution of methaneboronic acid in dried acetone (c = 5 mg/ml) and the mixture was kept at room temperature for 15 min.
2 µl were injected to GC/MSD.

Preparation of methyl derivatives: To the dried residue was added 250 µl acetone, 50 µl methyliodide and 100 mg K_2CO_3. The sample was vortex mixed and heated for 3 h at 60°C.

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2-3 µl were injected into GC/MSD.

Instrumental analysis

GC parameters:

Hewlett-Packard Gas Chromatograph, model 5890 serie II, NP Detector

column: HP Ultra -2, 17 m x 0.25 mm i.d., 0.32 µm film thickness

temperatures: injector -280°C, detector - 300°C and oven: initial 100°C - 2 min, final 290°C and rate of 20°C/min.

Carrier gas: Nitrogen - 1.12 ml/min., auxiliary -nitrogen at 28.5 ml/min, hydrogen: 4 ml/min, air-80 ml/min, split ratio: 1:10

GC/MS parameters:

HP 5890A GC coupled with HP 5970 MSD

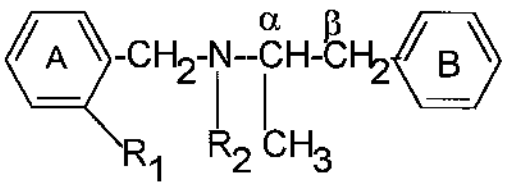
column: HP -5, 17 m x 0.25 mm i.d., 0.25 µm film thickness

temperatures: injector -280°C, transfer line - 280°C and oven: initial 110°C - 1 min, final 300°C and rate of 15°C/min.

Carrier gas: Helium - 1.3 ml/min, split ratio: 1:10

RESULTS AND DISCUSSION

The molecules of benzphetamine and clobenzorex have a close structure and built up of two aromatic rings and an aliphatic chain containing secondary amino group (I), in this connection was interested to be investigate and compare the metabolism of both substances.



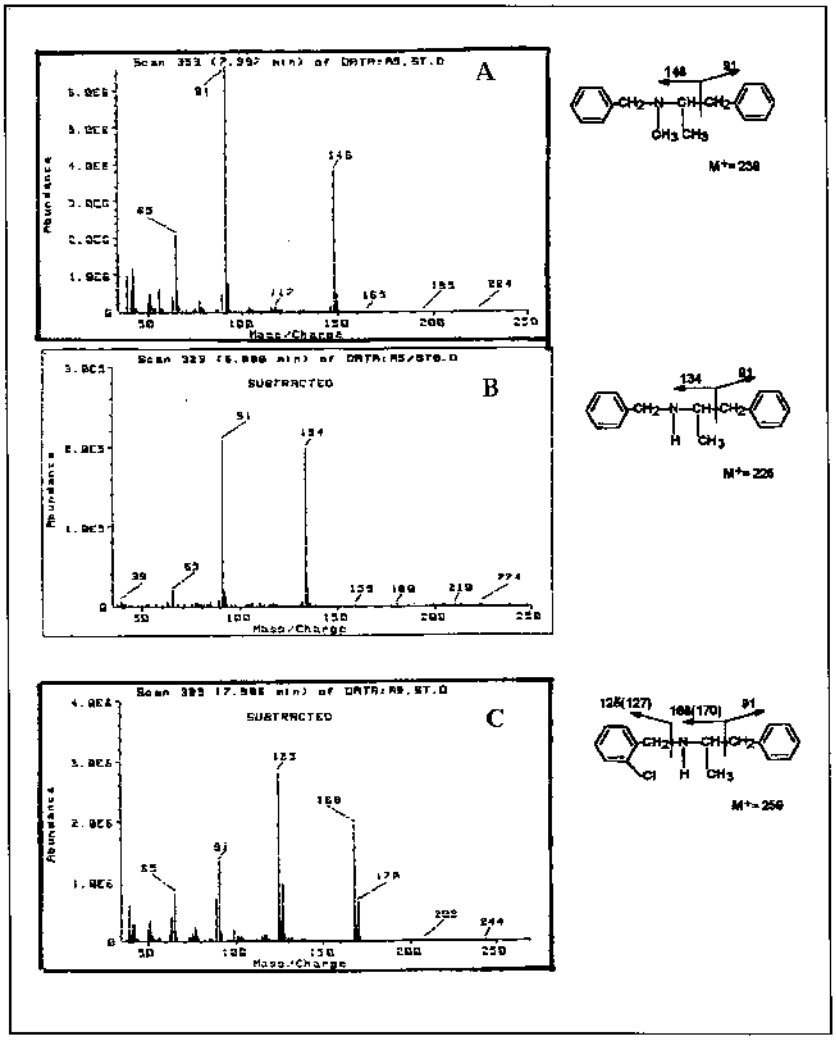
I

where: R_1=H, R_2=CH_3 for benzphetamine

R_1= Cl, R_2= H for clobenzorex

Their chemical structures permit the formation of great number of likely metabolites in the body receiving by demethylation, hydroxylation, O-methylation and/or splitting of the molecule. It is known that CYP3A4 enzyme catalyses N-demethylation of benzphetamine [2]. The hydroxylation following by O-methylation is possible also and can be performed on different places (para- or meta-) in the both aromatic rings - A or B and/or in β - position of the aliphatic chain.

On the Fig. 1 can be seen the electron impact (EI) fragmentations of benzphetamine and clobenzorex. Their similarity is based on their closed chemical structures. The mass spectra of both compounds have not representative molecular ions (M^+) but they possess intensive ion at m/e 91 resp. m/e 125 (127). The characteristic ions at m/e 148 for benzphetamine and 168 (170) for clobenzorex are dominating and formed from the cleavage of tropilium ion from M^+. The common specificity in the fragmentation and the presence of the chlorine atom in the chemical structure of clobenzorex have assisted for the following identifications of the found metabolites in studied urine samples.



The performed GC analyses of urine samples treated with usual procedure for free stimulants were confirmed the presence of known metabolites for benzphetamine [3] - amphetamine, methamphetamine and desmethylbenzphetamine and only amphetamine for clobenzorex. The gas chromatograms of the blank and studied urine samples are illustrated on Fig. 2.

Fig.1. Mass spectra of underivatized Benzphetamine (A), Desmethylbenzphetamine (B) and Clobenzorex (C).

The excretions of amphetamine and methamphetamine in urine samples were studied and the obtained results are shown on the Fig. 3. It is interesting to note that maximums on the excretion curves of amphetamine and methamphetamine are registered at about 12 hours after

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administration of benzphetamine while the maximum of amphetamine excretion after the application of clobenzorex is at the 6th hours. The investigations were confirmed the presence of desmethylbenzphetamine in lower level. The EI fragmentation of desmethylbenzphetamine (Fig. 1c) is very closed to the benzphetamine.

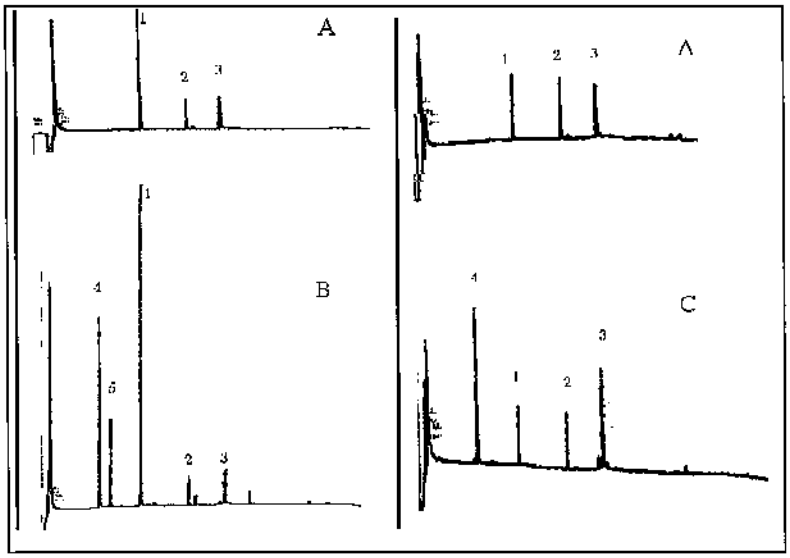

Fig.2. *GC/NPD chromatograms of blank urine (A) and urine sample after application of benzphetamine (B) resp. clobenzorex (C). The recorded peaks are: 1- nicotine; 2- ISTD -DPA; 3- caffeine; 4- amphetamine and 5- methamphetamine.*

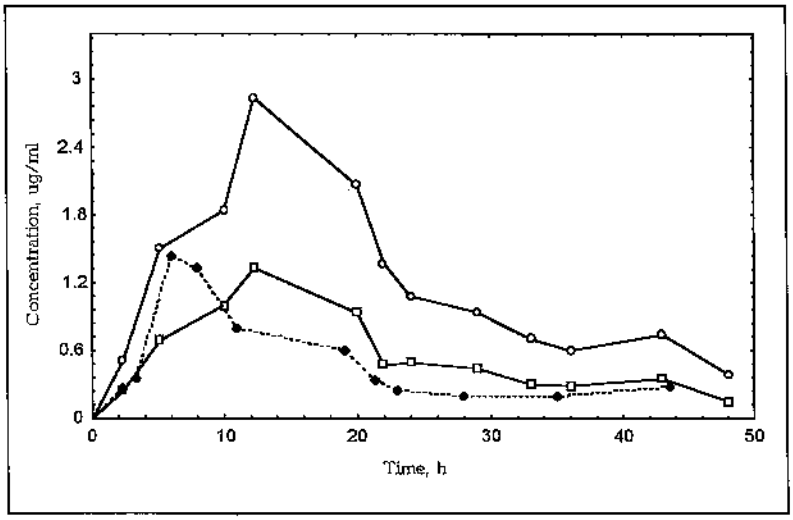

Fig.3. *Excretion of Amphetamine (-o-); Methamphetamine (--□--) after oral administration of Benzphetamine and Amphetamine (◆) after application of Clobenzorex.*

Analysing the urine samples under conditions for free stimulants were not found unchanged started substances or other metabolites. For this reason a set of experiments was carried out after hydrolyses of urine samples with Helix Pomatia. The obtained data from performed GC/MS analyses confirm the presence of conjugated metabolites of benzphetamine and clobenzorex as would be expected. The identification of the found metabolites and the confirmation of their structures were enabled by using different kind of derivatisation -

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methylation, silyllation, selective derivatization (silyllation and acetylation) and cyclization with methaneboronic acid.

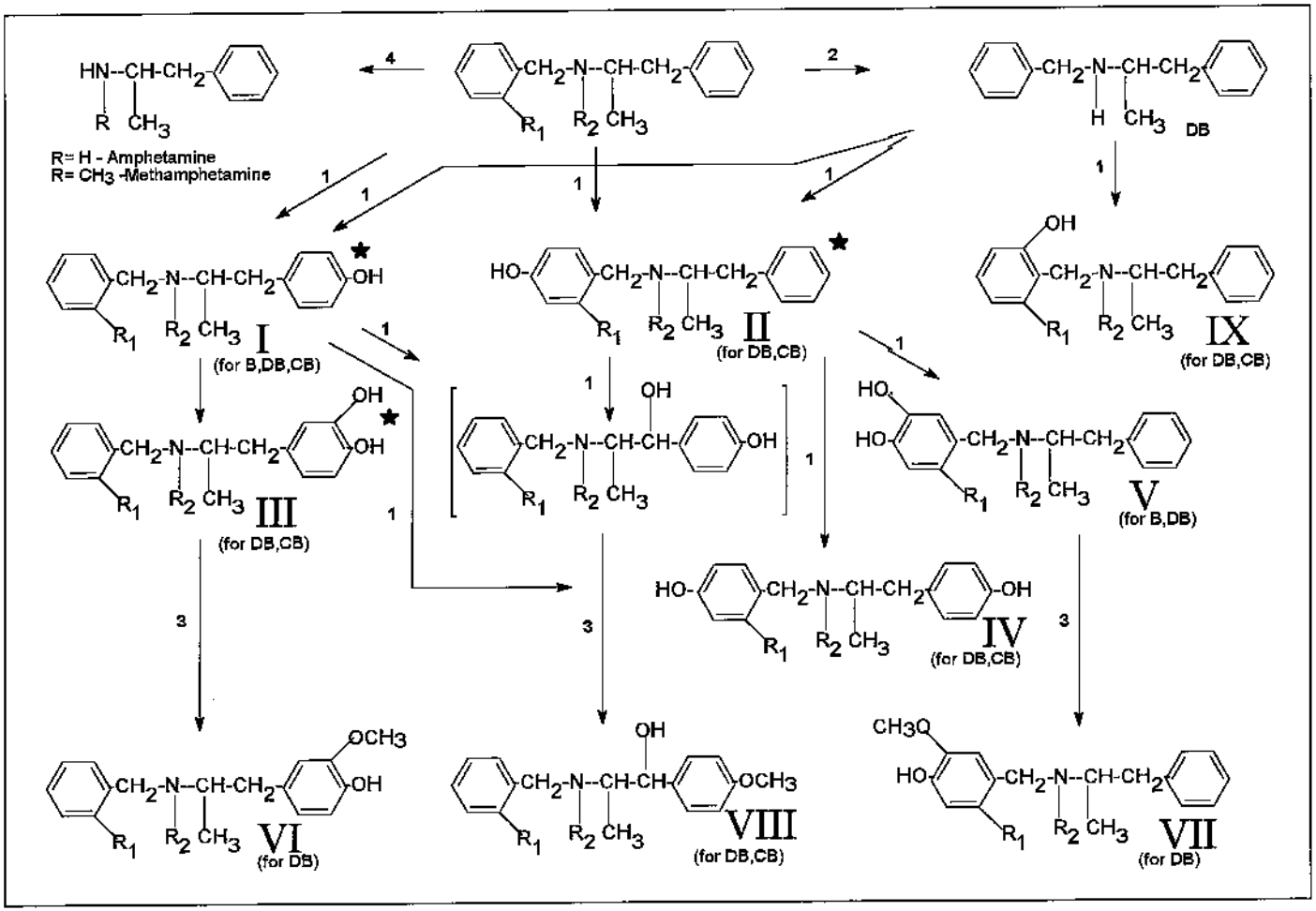
The probable metabolic pathway of benzphetamine and clobenzorex is based on the received results and presented on Fig. 4. The positions of hydroxy- and methoxy- groups in the metabolic structures are determined by interpretation of the obtained mass spectra from different derivatives and the corresponded retention times (Table 1). The most evident proofs for the structures were obtained by selective derivatization and cyclization by methaneboronic acid, that is why the next discussion concerns to these data.

Identification after selective derivatization

Monohydroxy metabolites

Figures 5 and 6 represent the mass spectra of both types of monohydroxymetabolites - I and II. It can be seen that they have common ions at m/e 91 resp. 125 (127) and similarly the specific ions formed according to the situation of the hydroxy group on A- or B-ring. On the other hand the compounds with the same type structure give a similar fragmentation. The presence of the ion at m/e 179 accompanied by specific ion at m/e 206 in the mass spectra of monohydroxy clobenzorex and desmethylbenzphetamine shows that the hydroxy group is situated on the outlying aromatic ring from the nitrogen atom (B-ring). The ions at m/e 230 (Fig. 5b) and m/e 264 (Fig. 5a) are formed by the cleavage of the ion 179 from M^+. The mass spectrum of hydroxybenzphetamine (Fig. 5c) confirms the position of hydroxy group in B-ring with the ions at m/e 236 (M^+- 91) and m/e 91, 148,179.

Figure 6 represents the mass spectra of another type of monohydroxymetabolites - Met. II which OH-group is disposed in A-ring. It can be seen that the basic ion is at m/e 213 (215) (Fig. 6a) resp. m/e 179 (Fig. 6b). The specific ion at m/e 325 (327) resp. m/e 291 produced by cleavage of the fragment $C_6H_5CH_2CH{=}CH_2$ from the M^+ (M^+ - 118) and the absent of ion at m/e 206 confirm the position of the OH-group on A-ring. The found quantity of Met. II for desmethylbenzphetamine is higher than for clobenzorex. It can be explained with the influence of chlorine atom in A-aromatic ring of clobenzorex under the hydroxylation.



R_1=Cl; R_2=H - Clobenzorex (CB)
R_1=H; R_2=CH_3 -Benzphetamine (B)
R_1=H; R_2=H -Desmethyl-benzphetamine (DB)
The major metabolites are pointed with ★.

Reaction
1- hydroxylation
2- N-demethylation
3- O-methylation
4- degradation

Fig.4 . Probable metabolic pathway of clobenzorex and benzphetamine

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Table 1

Relative retention times and M^+ of detected Metabolites of Benzphetamine and Clobenzorex in hydrolysed urine samples after different derivatization: silyllation(MSTFA), selective derivatization (MSTFA/MBTFA) and cyclization with MBA

Meta-bolites	Clobenzorex						Benzphetamine						Desmethylbenzphetamine					
	MSTFA		MSTFA/ MBTFA		MBA		MSTFA		MSTFA/ MBTFA		MBA		MSTFA		MSTFA/ MBTFA		MBA	
	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+	RRT	M^+
I	1.20	419	1.36	443	n.d.	-	1.17	327	1.20	327	n.d.	-	1.28	385	1.26	409	n.d.	-
II	1.14	419	1.27	443	n.d.	-	-	-	-	-	-	-	1.27	385	1.25	409	n.d.	-
III	1.34	507	1.39	531	* 1.06/ 1.11	315	-	-	-	-	-	-	1.40	473	1.39	497	* 0.95/ 0.98	281
IV	1.40	507	1.47	531	n.d.	-	-	-	-	-	-	-	1.48	473	1.47	497	n.d.	-
V	-	-	-	-	-	-	1.25	415	1.27	415	0.86	295	1.39	473	1.37	497	0.92	281
VI	-	-	-	-	-	-	-	-	-	-	-	-	1.35	415	1.33	439	n.d.	-
VII	-	-	-	-	-	-	-	-	-	-	-	-	1.37	415	1.35	439	n.d.	-
VIII	1.27	449	1.37	473	* 1.04/ 1.08	329	-	-	-	-	-	-	1.38	415	1.36	439	* 0.94/ 0.97	295
IX					0.82	265	-	-	-	-	-	-					0.82	265

Remarks: *1. Bamethane was used as ISTD with next retention times - Bamethane (2 O-TMS, N-TMS) t_R = 7.92 min.; Bamethane (2 O-TMS, N-TFA) - t_R = 7.76 min.*

2. MBA derivatization was carried out with the presence of Pindolol as ISTD with t_R = 9.29 min. as Pindolol-boronate.

3. The abbreviation n.d. denote that compound does not react with MBA.

4. After cyclization with MBA the marked couple of RRT with (*) correspond to the isomers of metabolites.

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Dihydroxy metabolites

Figure 7 represents the mass spectra of dihydroxymetabolites (Met. IV) of clobenzorex and desmethylbenzphetamine. The ion at m/e 91 resp. 125 (127) misses in both mass spectra. This fact confirms that both OH-groups are situated on A- and B-rings. In the mass spectrum of clobenzorex can be seen the ions at m/e 179, 206 and 213 (215). In the mass spectrum of desmethylbenzphetamine the ion at m/e 179 is more intensive than ion at m/e 206 (Fig. 7b). The quantity of dihydroxyclobenzorex is lower than those of desmethylbenzphetamine.

Figure 8 presents the mass spectra of another type of dihydroxymetabolites III and V that have two hydroxy groups situated on one of the rings. The intensive ions at m/e 267, 179 and the specific one for the structure of pattern substances (for clobenzorex -ion at m/e 125 (127) and for desmethylbenzphetamine - ion at m/e 91) confirm the above. The position of both hydroxy groups has established by specific ions as m/e 294 and 378. The ion at m/e 294 is formed by cleavage of the fragment $C_6H_5CH_2N$-TFA (for desmethylbenzphetamine) and $C_6H_4ClCH_2N$-TFA (for clobenzorex) from M^+ and is characteristic for OH groups on the B ring (Met. III). When OH groups are situated on ring A (Met. V) the cleavage of fragment $C_6H_5CH_2CH{=}CH_2$ (M^+ - 118) leads to formation of the ion at m/e 378 (Fig.8c). This way of fragmentation is similarly to that of Met. II (Fig. 6). Dihydroxybenzphetamine which hydroxy groups are on A-ring was detected in negligible quantity.

Hydroxy-, methoxy- metabolites

Figure 9 presents two mass spectra of O-TMS, N-TFA methoxydesmethyl-benzphetamine (Mets. VI and VII). The specific ion at m/e 209 gives a proof that O-TMS, OCH_3 -groups are situated on the one of aromatic rings. The presence of intensive ion at m/e 91 and ion at m/e 179 (formed by a cleavage of OCH_3 group from the ion at m/e 209) are in according with above statement. The ions at m/e 236 and m/e 320 have determined the structural difference between two compounds. The specific ion at m/e 236 had formed by cleavage of fragment C_6H_5 CH_2N-TFA from M^+ when the O-TMS, OCH_3 - groups are situated on B-ring (Met. VII), while the ion at m/e 320 is specific for A-ring disposition of groups (Met. VI). This ion at m/e 320 is produced by cleavage of fragment $C_6H_5CH_2CH{=}CH_2$ from M^+ (Fig. 9b).

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Identification after cyclization with methaneboronic acid

It is known that 1,2- or 1,3- aromatic- or aliphatic- diols and 1,2- or 1,3- aminoalcohols react with some of alkyl- and aryl boronic acids and the obtained boronate derivatives are stable compounds with good chromatographic properties. Their mass spectra are specific and give the information about the neighbourhood of two hydroxy- or hydroxy- and amino- groups [4-7]. The structures of the discussed above Mets. III and V were confirmed by mass spectra received after cyclization with methaneboronic acid. Figure 10 shows the mass spectra of both types of dihydroxymetabolites which have different fragmentation. The positions of both hydroxy groups on B-ring determinate specific the fragments at m/e 91, resp. 125 (127) and 160 (Fig. 10 a, b), while the position on A-ring - m/e 147, 91 and 190 (for dihydroxydes-methylbenzphetamine - Fig. 10c) resp. m/e 204 for dihyroxybenzphetamine.

The derivatization with methaneboronic acid has assisted for identification of another hydroxy- methoxy-metabolite (Mets. VIII) of clobenzorex and desmethylbenzphetamine. The hydroxy group is situated in β-position of the aliphatic chain and methoxy one is on B-ring. The specific ions are m/e 91 respectively 125 (127) and 173, 174 (Fig. 11a, b).

On Fig. 11c can be seen the mass spectrum of boronate derivative that is received from urine samples of benzphetamine and clobenzorex also. We assume that the metabolite IX is monohydroxy with hydroxy group in *ortho*-place on A-ring. The detection of the such metabolite at the same retention time from the urine sample of clobenzorex may be explain by a possible dehalogenation of the molecule.

Analysing the urine samples after reaction by methaneboronic acid were detected some couples of peaks with close retention times and equal spectra corresponding to those of Mets. III and VIII. According to the reported data [7,8] the methyl- or butyl- boronate derivatives of structural- and stereo- isomers can be separated under GC analysis on achiral stationary phases (OV-1, OV-17). The obtained data give us the reason to assume that these detected compounds are structural- or stereo-isomers.

CONCLUSION

Several new metabolites of benzphetamine and clobenzorex are established as a result of the conducted investigation. The structures of these compounds are discussed and the probable metabolism scheme is proposed. The mono- and dihydroxy- metabolites of the studied drugs are the major compounds excreted as conjugated in the urine.

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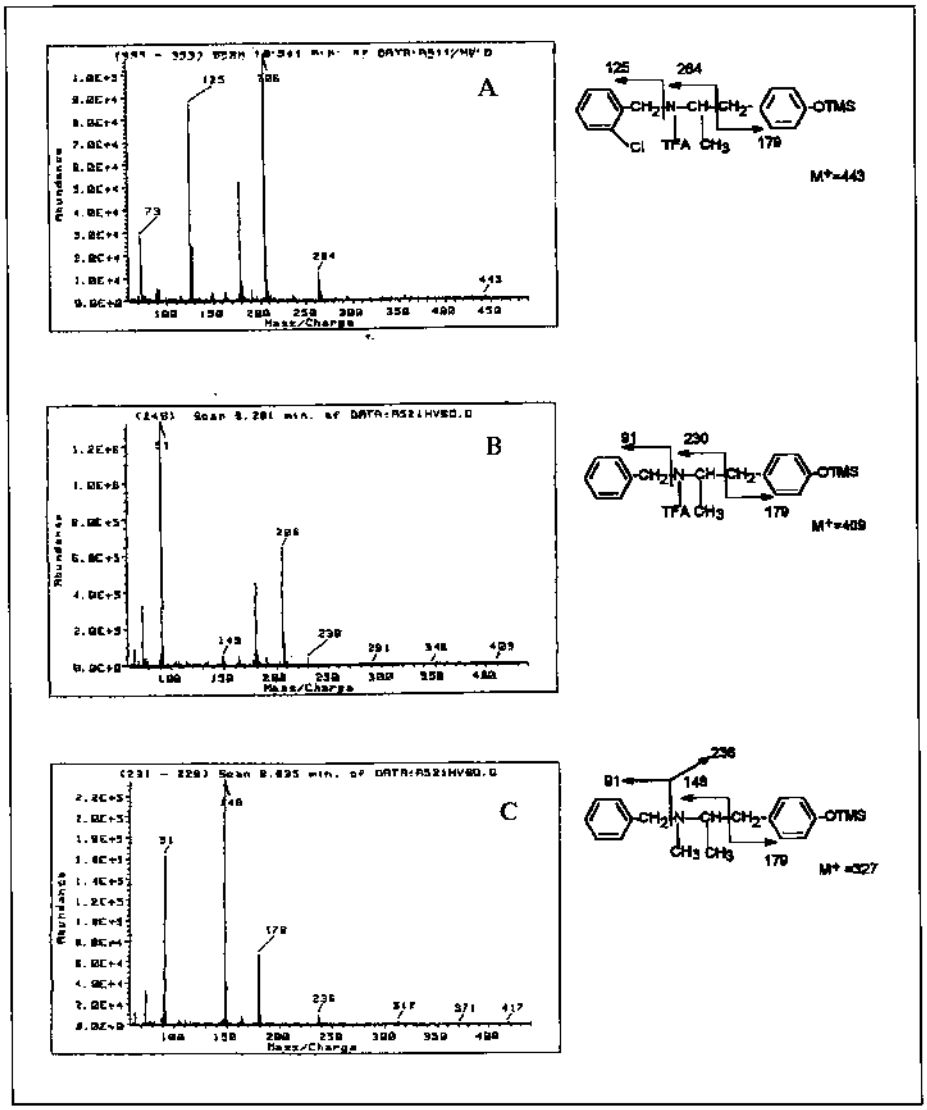

Fig.5. *Mass spectra of the monohydroxy metabolites* ***I*** *of clobenzorex (A), desmethylbenzphetamine (B) and benzphetamine (C) after derivatisation with MSTFA/MBTFA.*

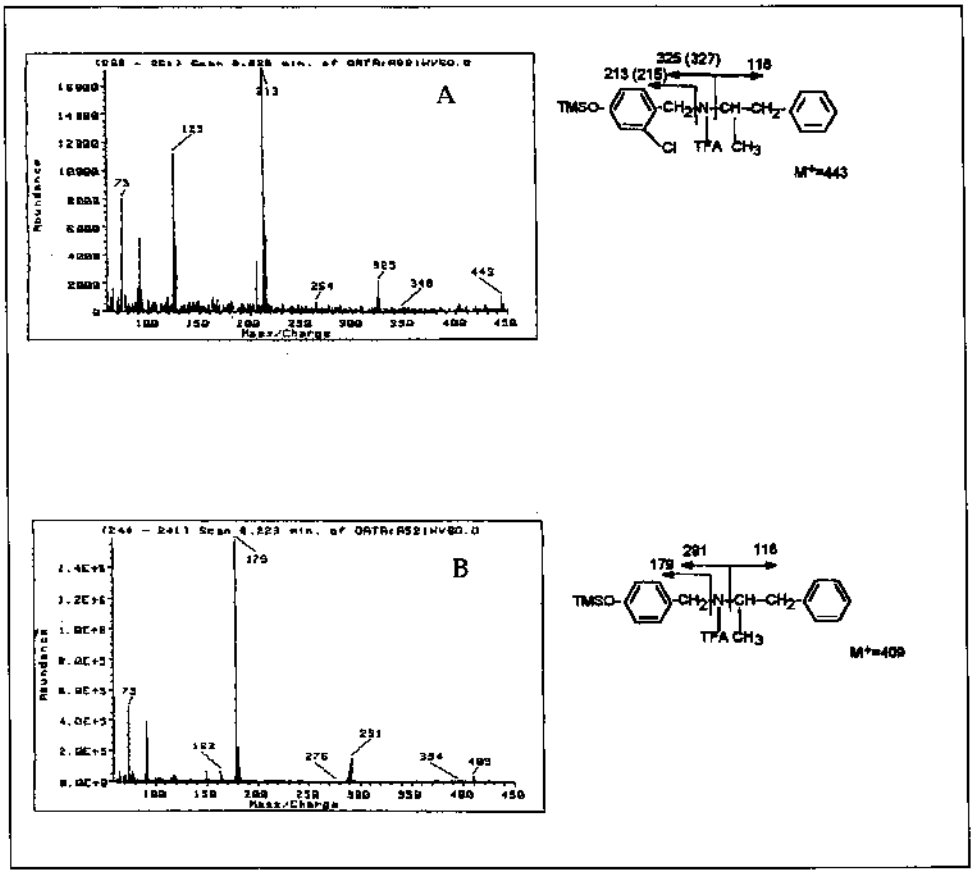

*Fig. 6. Mass spectra of the monohydroxy metabolites **II** of clobenzorex (A) and desmethylbenzphetamine (B) afer derivatisation with MSTFA/MBTFA.*

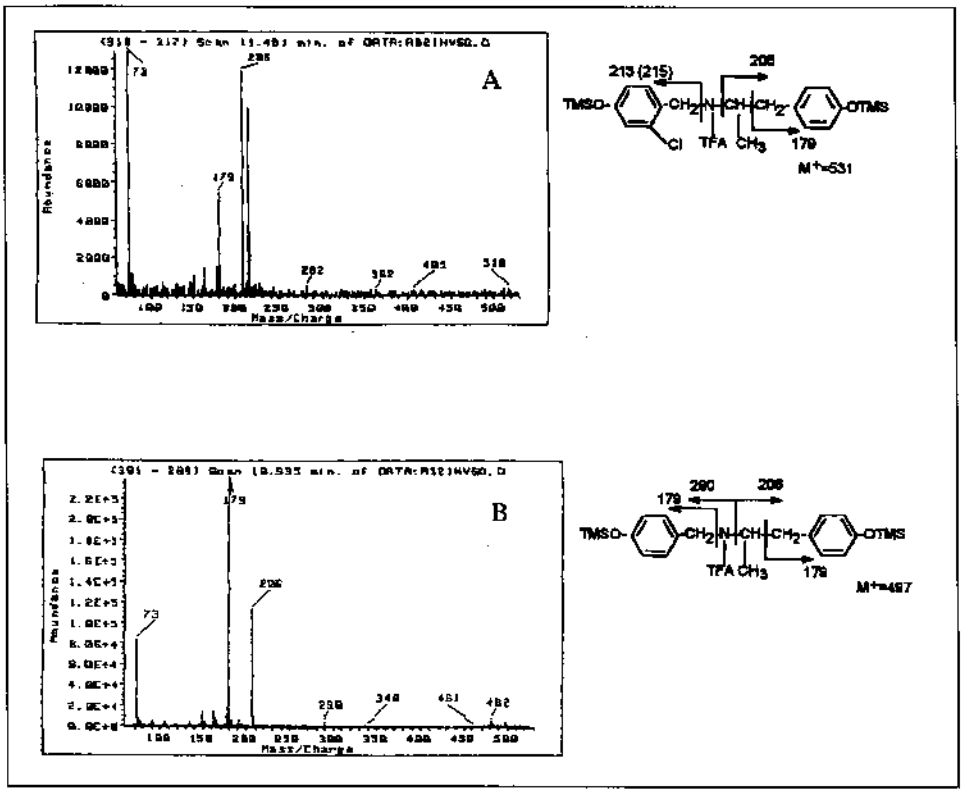

*Fig. 7. Mass spectra of the dihydroxy metabolites **IV** of clobenzorex (A) and desmethylbenzphetamine (B) after derivatisation with MSTFA/MBTFA.*

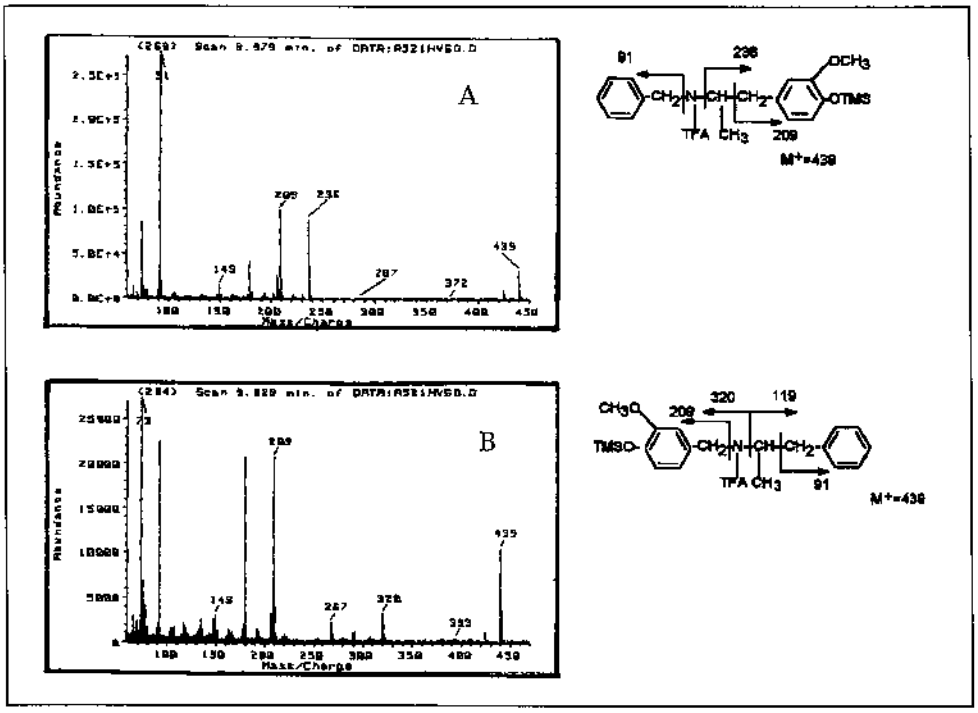

*Fig. 9. Mass spectra of the hydroxy- methoxy metabolites of desmethylbenzphetamine **VI** (A) and **VII** (B) after derivatisation with MSTFA/MBTFA.*

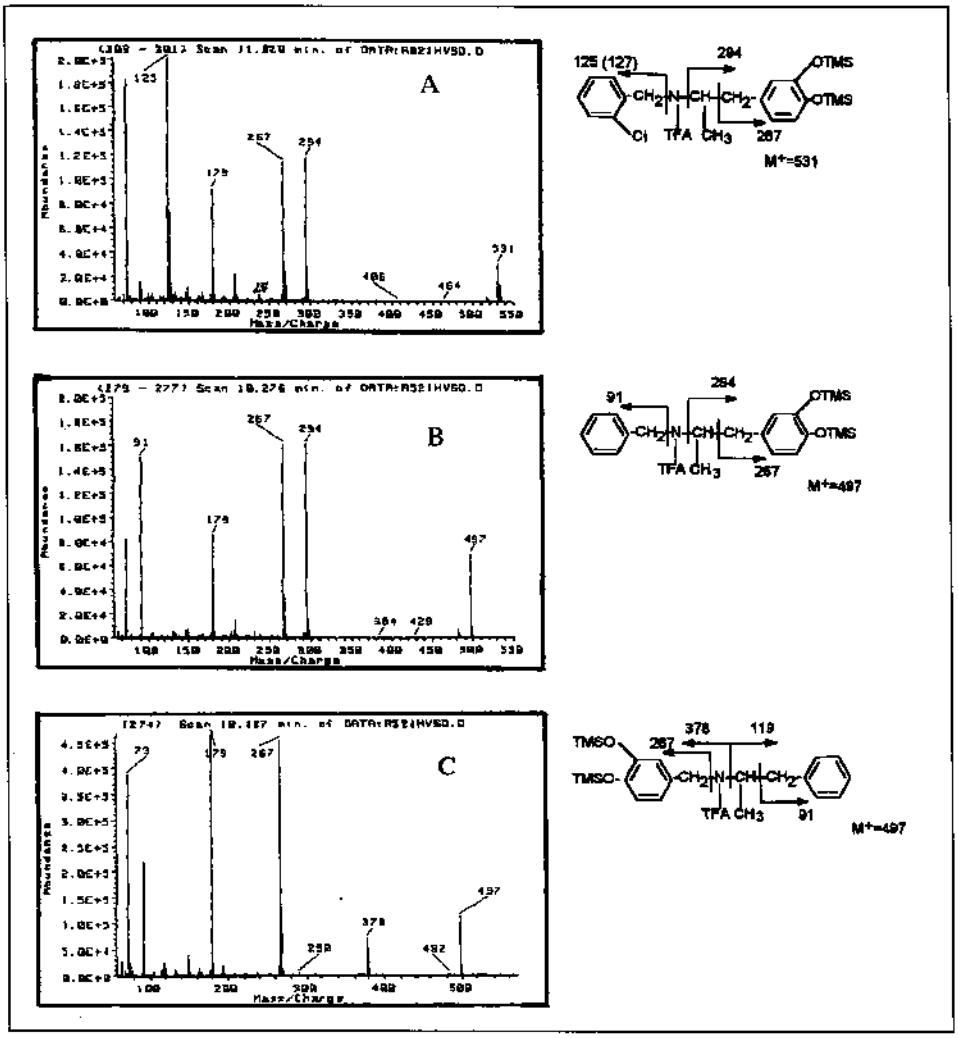

Fig. 8. *Mass spectra of the dihydroxy metabolites* ***III*** *of clobenzorex (A), desmethylbenzphetamine (B) and dihydroxymetabolite* ***V*** *of desmethylbenzphetamine (C) after derivatisation with MSTFA/MBTFA.*

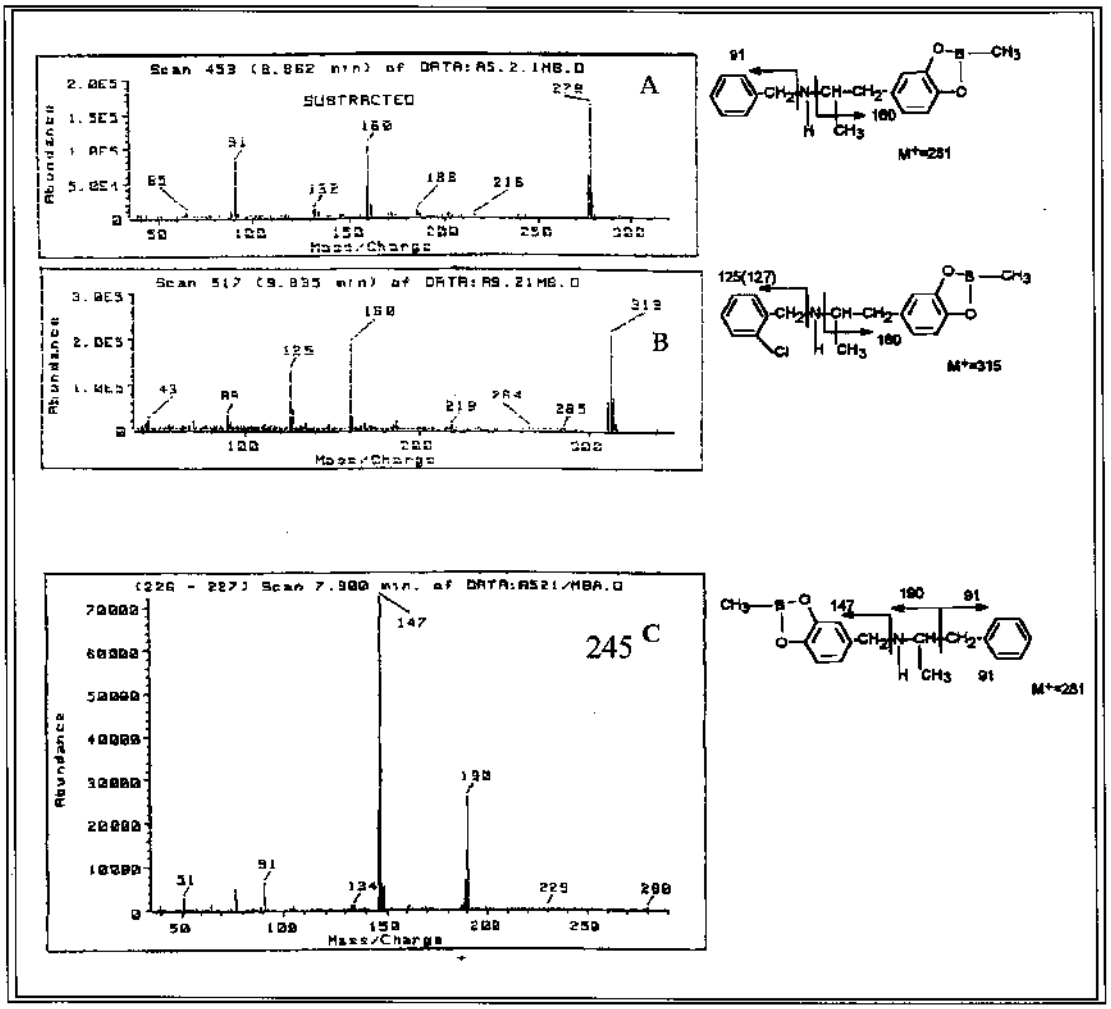

Fig.10. *Mass spectra of the dihydroxy metabolites* ***III*** *of desmethylbenzphetamine (A) clobenzorex (B) and dihydroxymetabolite* ***V*** *of desmethylbenzphetamine (C) after derivatisation with MBA.*

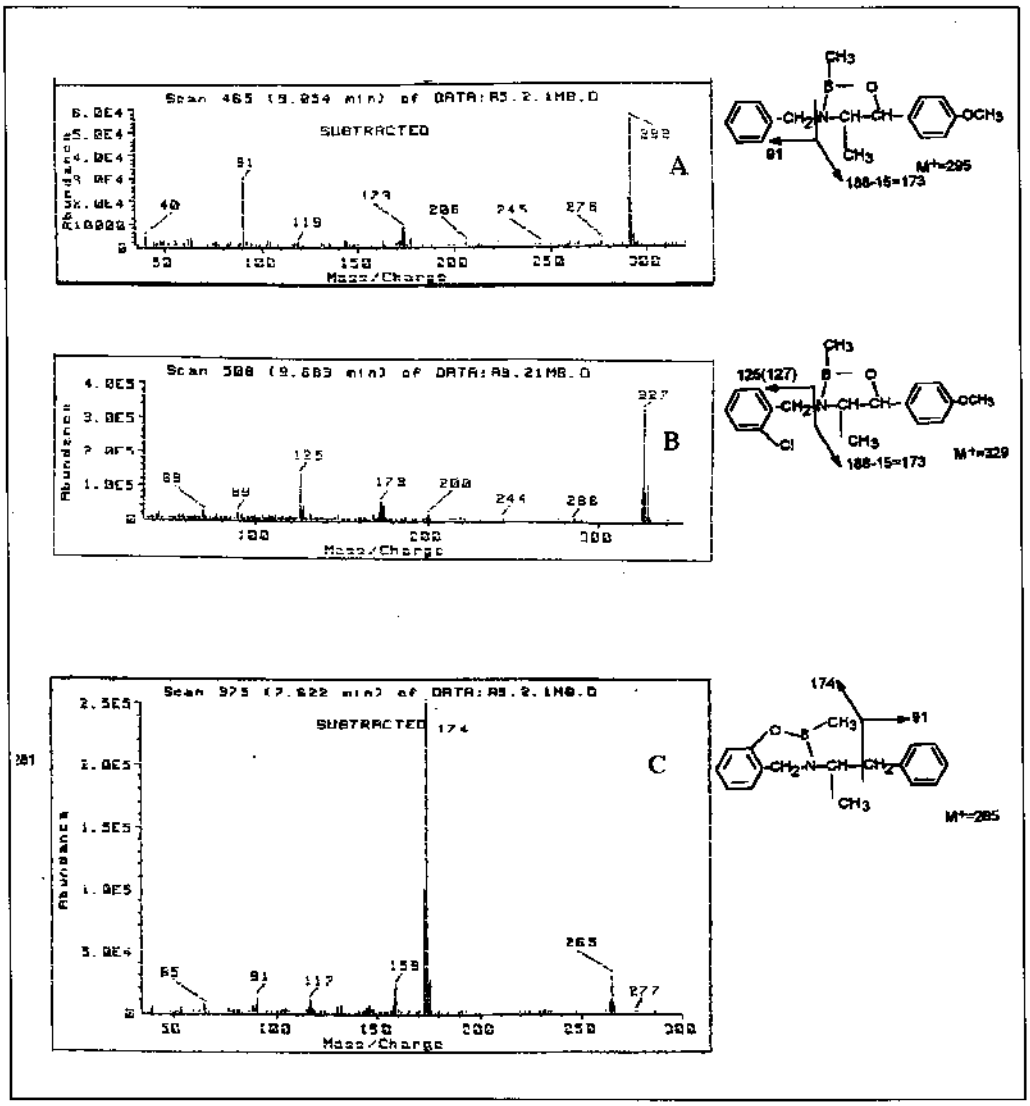

Fig.11. *Mass spectra of the hydroxy-methoxy metabolites* ***VIII*** *of desmethylbenzphetamine (A) clobenzorex (B) and monohydroxymetabolite* ***IX*** *of desmethylbenzphetamine (C) after derivatisation with MBA.*

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Amphetamine, Clobenzorex, and 4-Hydroxyclobenzorex Levels Following Multidose Administration of Clobenzorex

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Abstract

Clobenzorex (Asenlix) is an anorectic drug used as part of a weight-management program. The drug is metabolized by the body to amphetamine, which is then excreted in the urine, thus causing difficulty in interpretation of amphetamine-positive drug tests. Previous studies have shown that the parent drug and several metabolites are excreted in urine. Clobenzorex itself has been detected for as long as 29 h following administration of a single dose. However, the parent drug was not always detected in samples that contained amphetamine at ≥ 500 ng/mL, the administrative cutoff for a positive result. Consequently, the parent compound clobenzorex is not ideal for ascertaining whether the drug was the origin of the amphetamine. Several metabolites of clobenzorex have been shown to be detected for a longer period of time than the parent. One of these, a hydroxy metabolite, was shown to be detected for an extended period of time. In a study of urine samples provided following administration of a single 30-mg dose of this drug, 4-hydroxyclobenzorex could be detected for up to 91.5 h. More significantly, that study showed all samples that were positive for amphetamine also contained detectable amounts of 4-hydroxyclobenzorex. This metabolite proved to be easily detected and was typically found at higher levels than amphetamine in urine samples positive for amphetamine long after clobenzorex itself could no longer be detected. The present study analyzed samples from a controlled multidose administration (30 mg of clobenzorex daily for seven days) for the presence of 4-hydroxyclobenzorex. The analytical procedure used acid hydrolysis followed by liquid–liquid extraction and gas chromatographic–mass spectrometric analysis with monitoring of ions at *m/z* 125, 330, and 364 for 4-hydroxyclobenzorex and its 3-Cl regioisomer, which was used as an internal standard. Peak concentrations of 4-hydroxyclobenzorex ranged from 17,786 to 99,044 ng/mL. Most importantly, this study also found that all samples that contained amphetamine at ≥ 500 ng/mL also contained detectable amounts of this hydroxy metabolite (LOD 10 ng/mL), making it a valuable tool in differentiating use of clobenzorex from illicit amphetamine use.

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Introduction

Clobenzorex, *N*-[2-chlorobenzyl]-amphetamine, is an anorectic drug that, although not sold in the United States, is available in many countries as a prescription (e.g., Mexico) or over-the-counter (e.g., Panama) drug. The recommended starting dose is 30 mg (one capsule) per day. It is one of several so-called "precursor drugs" that are metabolized to amphetamine by the body and excreted in the urine. Because it is metabolized to amphetamine, therapeutic use of clobenzorex may lead to suspicion of amphetamine abuse. Drugs that are metabolized to methamphetamine and/or amphetamine are potentially significant concerns in the interpretation of amphetamine-positive drug testing results. The presence of the parent compound or a unique metabolite can be strong evidence for the involvement of this drug as opposed to amphetamine abuse.

An accurate and reliable quantitative gas chromatography–mass spectrometry (GC–MS) selected ion monitoring method for clobenzorex using a regioisomer as the internal standard has previously been developed. The method has a limit of detection (LOD) and limit of quantitation (LOQ) for clobenzorex at 1 ng/mL (1,2). However, even with this low LOD, the parent compound was not always detected in samples positive (≥ 500 ng/mL) for amphetamine (1). In view of this fact, it appeared that evaluation of a metabolite might prove to be a better indicator of clobenzorex use. Several metabolites, including two different hydroxy metabolites, have previously been identified (3). In a later study involving the administration of a single 30-mg dose of clobenzorex, one of these hydroxy metabolites, 4-hy-

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droxyclobenzorex (4-HO-clobenzorex), was detected in all amphetamine-positive samples (4). The present study extends that information to delineate detection times and concentrations seen when the drug is taken repeatedly rather than as a single dose.

Materials and Methods

Materials

Amphetamine, methamphetamine, amphetamine-d_6 (1-phenyl-2-aminopropane-1,1,2,3,3,3-d_6), and methamphetamine-d_{11} (1-phenyl-d_5-2-methyl-d_3-aminopropane-3,3,3-d_3) were obtained from Radian Corp. Clobenzorex hydrochloride was obtained through colleagues in Spain. The internal standard, *N*-[3-chlorobenzyl]-amphetamine (3-Cl-clobenzorex), was synthesized in our laboratory following the procedure described by Lintermans et al. (5). The standard, 4-HO-clobenzorex, and the internal standard, *N*-[3-chlorobenzyl]-4-hydroxyamphetamine (3-Cl-4-HO- clobenzorex), were obtained from Isotech, Inc. Heptafluorobutyric anhydride (HFBA) was obtained from Sigma Chemical Co. Clobenzorex administered to experimental subjects in the form of Asenlix (Roussel, Mexico) was purchased from a pharmacy in Mexico. Structures of clobenzorex, its hydroxy metabolite, and their internal standards are shown in Figure 1.

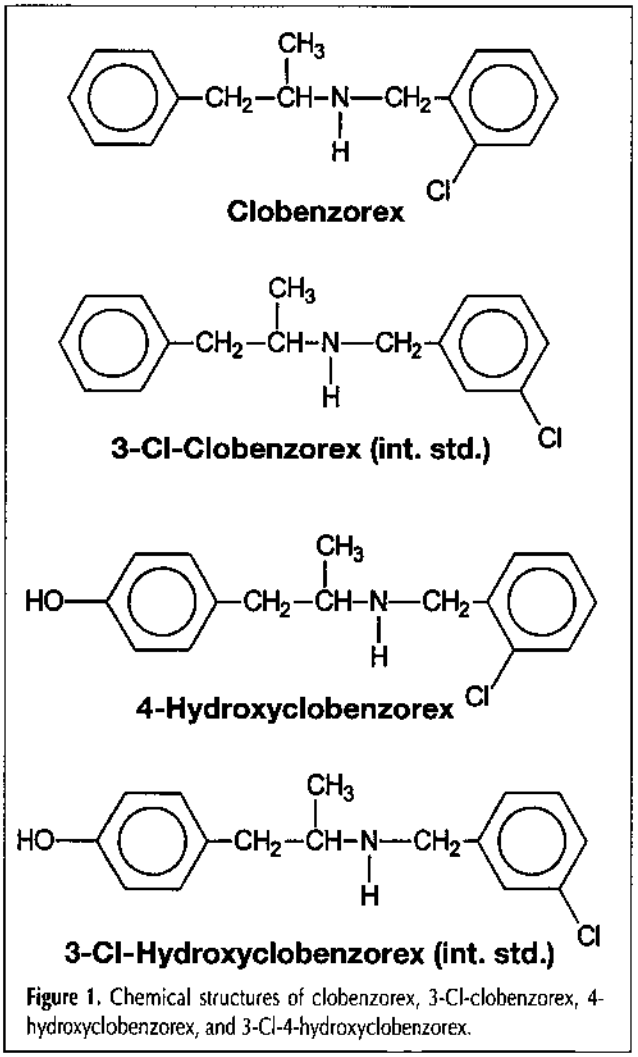

Figure 1. Chemical structures of clobenzorex, 3-Cl-clobenzorex, 4-hydroxyclobenzorex, and 3-Cl-4-hydroxyclobenzorex.

Drug administration and sample collection

Thirty milligrams of clobenzorex, in the form of a single Asenlix capsule, was administered orally for seven consecutive days to five healthy volunteers (three female and two male) with no history of amphetamine use. A predose sample was taken prior to drug administration and, following the first administration of the drug, urine was collected for the next 14 days. Samples were provided ad lib and refrigerated or frozen until analysis. No attempt was made to physiologically control urine pH during this study.

Sample preparation and analysis

Sample pH was measured using a Fisher Accumet 50 pH meter, and specific gravity was determined using an AO Scientific Instruments refractometer. Creatinine levels were determined at the Wilford Hall Medical Center clinical laboratory using standard clinical laboratory procedures.

Hydrolysis was accomplished by addition of 1 mL 30% hydrochloric acid to 2 mL of urine. The samples were capped loosely, vortex mixed, and then placed in an autoclave (121°C, 15 psi) for 30 min. Samples were removed, allowed to cool to room temperature, and then extracted and derivatized as previously described (1,4).

GC–MS analyses were performed using a Hewlett-Packard 6890 II GC coupled with an HP 5973 MS using a 7683 autoinjector with minor modification to previously described procedures (1,4). Chromatography was accomplished using either an HP-1 capillary column (Hewlett Packard, 12 m × 0.2-mm i.d., 0.33-µm film thickness) or a ZB-50 column (Phenomenex, 15 m × 0.25-mm i.d., 0.25-µm film thickness). A temperature program with an initial time of 1 min at 80°C, programmed to 180°C at 20°C/min, and then to 250°C at 28°C/min with a 1 min final time was used with either column. Quantitation was based on single-point calibration using a calibration standard containing 500 ng/mL of amphetamine, methamphetamine, and 4-HO-clobenzorex with 250 ng/mL of deuterium-labeled isotopomer internal standards for amphetamine and methamphetamine and 250 or 500 ng/mL of 3-Cl-4-HO-clobenzorex. Clobenzorex can also be analyzed with this assay by adding 25 ng/mL clobenzorex and 50 ng/mL of 3-Cl-clobenzorex as internal standard to the calibrator. Use of 250 or 500 ng/mL internal standard for the quantitation of 4-HO-clobenzorex gave comparable linearity. LODs for this assay were 5 ng/mL for amphetamine and methamphetamine, 1 ng/mL for clobenzorex, and 10 ng/mL for 4-HO-clobenzorex. The LOQ was 5 ng/mL for amphetamine and methamphetamine, 1 ng/mL for clobenzorex, and 25 ng/mL for 4-HO-clobenzorex. The upper limit of linearity for the method is 10,000 ng/mL for amphetamine, methamphetamine, and 4-HO-clobenzorex and 100 ng/mL for clobenzorex. Ions monitored were *m/z* 240, 118, and 91 (amphetamine); *m/z* 254, 210, and 118 (methamphetamine); *m/z* 244 and 123 (amphetamine-d_6); *m/z* 260 and 213 (methamphetamine-d_{11}); *m/z* 118, 125, and 364 (clobenzorex and 3-Cl-clobenzorex); and *m/z* 125, 330, and 364 (4-HO-clobenzorex and 3-Cl-4-HO-clobenzorex).

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Table 1. Analytical Results Following Multidose Administration of Clobenzorex*

Sample	pH	Sp. gr.†	Creatinine	Hours post-first dose	Concentration (ng/mL) Amp	Clob	4-HO-clob
Subject 1							
Pre - 1	5.17	1.005	33.9	00:00	0	0.0	0
2	5.12	1.004	24.8	00:25	0	0.0	0
3	7.28	1.010	44.3	03:00	224	2.5	13,001
4	6.32	1.012	80.6	08:04	**1025**	4.1	7184
5	6.14	1.015	111.2	10:15	**764**	1.0	1093
6	6.03	1.004	30.1	11:00	472	0.0	202
7	6.11	1.011	79.6	11:50	**560**	0.0	711
8	6.59	1.009	66.7	13:35	289	0.0	410
9	5.43	1.016	160.6	22:30	**1314**	0.0	436
10	6.69	1.011	73.3	29:25	**926**	4.1	16,241
11	6.03	1.005	60.5	31:10	**683**	1.3	2390
12	6.62	1.007	61.3	34:00	**509**	0.0	987
13	7.17	1.011	89.7	36:30	281	0.0	747
14	6.77	1.004	33.8	40:15	408	3.9	136
15	7.27	1.007	57.6	40:50	211	1.8	114
16	6.68	1.010	93.4	42:30	466	2.0	223
17	7.43	1.016	130.8	47:00	151	0.0	239
18	7.43	1.004	21.8	49:20	99	0.0	287
19	7.77	1.010	65.3	51:37	126	5.0	19,681
20	5.08	1.014	134.8	53:35	**3065**	42.0	33,028
21	6.40	1.018	232.4	55:25	**1639**	4.6	19,316
22	6.04	1.021	242.3	59:30	**3360**	3.8	4631
23	6.87	1.008	58.4	60:25	437	0.0	437
24	6.91	1.011	76.3	62:20	489	0.0	669
25	5.83	1.019	195.3	67:10	**1954**	0.0	1088
26	5.20	1.020	276.6	71:45	**2970**	1.5	683
27	5.49	1.005	52.8	73:15	**1620**	5.8	25,444
28	5.48	1.005	46.8	74:34	**1724**	4.7	12,554
29	5.58	1.005	40.4	76:45	**1039**	1.6	1664
30	5.05	1.018	312.5	81:50	**4716**	6.8	6066
31	5.34	1.025	216.8	86:35	**3685**	1.5	2437
32	5.33	1.020	147.9	94:25	**1829**	1.5	638
33	5.70	1.006	54.5	97:40	**639**	3.3	5714
34	5.52	1.005	52.5	101:25	**1625**	6.4	9442
35	5.79	1.003	30.7	103:05	**771**	1.0	383
36	5.25	1.012	110.2	105:30	**2167**	2.6	914
37	5.33	1.006	81.5	108:20	**1361**	1.3	1053
38	4.90	1.008	79.2	110:00	**1623**	2.0	1311
39	5.14	1.011	123.5	117:25	**1390**	0.0	600
40	5.29	1.020	253.1	119:20	**1296**	1.2	1697
41	6.76	1.005	179.3	135:05	411	0.0	1270
42	6.49	1.021	348.8	138:20	**823**	0.0	917
43	7.50	1.015	161.9	142:25	100	0.0	215
44	7.05	1.008	78.3	148:41	**722**	2.7	38,832
45	6.63	1.017	201.9	152:10	**1643**	3.3	5766
46	6.38	1.005	64.3	153:20	**911**	1.0	543
47	6.99	1.003	19.5	155:00	256	0.0	283
48	7.26	1.004	26.9	155:46	165	0.0	133
49	5.65	1.014	194.2	166:35	**1920**	0.0	1231
50	5.27	1.018	177.9	172:30	**1797**	1.2	533
51	7.54	1.012	87.8	176:35	**701**	0.0	108
52	6.66	1.021	411.3	181:45	**2836**	1.4	446
53	6.65	1.015	167.1	190:35	**882**	0.0	94
54	6.56	1.004	39.0	202:00	72	2.0	Detected
55	6.93	1.002	14.2	204:00	21	0.0	0
56	5.99	1.007	66.8	204:10	162	1.4	56
57	7.19	1.006	39.8	205:55	29	0.0	0
58	6.50	1.015	143.2	216:16	107	0.0	35
59	5.54	1.015	113.7	217:42	157	0.0	Detected
60	5.11	1.015	120.7	218:30	214	0.0	Detected
61	5.11	1.017	191.5	221:25	322	0.0	31
62	5.26	1.022	193.3	224:22	267	0.0	Detected
63	5.29	1.024	240.8	227:20	279	0.0	Detected
64	5.53	1.011	83.3	230:40	74	0.0	0
65	5.54	1.015	141.0	238:00	81	0.0	Detected
66	5.53	1.020	208.5	242:08	78	0.0	Detected
67	5.55	1.013	111.9	246:45	31	0.0	Detected
68	5.27	1.023	309.6	249:35	79	0.0	Detected
69	5.61	1.005	37.5	251:40	13	0.0	0
70	5.58	1.010	88.1	253:00	18	0.0	0
71	5.72	1.011	100.3	258:30	18	0.0	0
72	5.50	1.020	229.3	262:50	33	0.0	0
73	6.00	1.006	62.8	264:25	0	0.0	0
74	6.98	1.014	121.2	268:30	0	0.0	0
75	6.03	1.021	200.2	274:21	13	0.0	0
76	6.03	1.016	146.8	277:26	13	0.0	0
77	5.86	1.008	58.4	289:10	0	0.0	0
78	6.67	1.008	45.9	291:38	0	0.0	0
79	6.21	1.015	118.3	296:21	0	0.0	0
80	6.99	1.008	55.1	297:03	0	0.0	0
81	6.79	1.004	25.2	297:53	0	0.0	0
82	6.90	1.007	39.6	299:04	0	0.0	0
83	9.71	1.011	75.2	301:30	0	0.0	0
84	5.76	1.014	159.7	310:39	8	0.0	0
Subject 2							
Pre - 1	5.21	1.019	42.1	00:00	0	0.0	0
2	5.73	1.008	42.6	04:00	**773**	4.4	13,684
3	7.15	1.018	147.8	08:00	**1207**	2.6	7512
4	6.89	1.022	159.1	13:30	**1436**	0.0	1322
5	6.14	1.021	132.0	20:30	**2158**	0.0	711
6	6.31	1.019	100.1	26:00	**1529**	5.3	15,741
7	7.24	1.004	77.8	31:30	**1777**	2.2	5593
8	7.63	1.005	24.6	33:30	399	0.0	526
9	6.29	1.007	49.2	37:30	**1812**	0.0	437
10	5.75	1.016	119.1	44:30	**2079**	1.1	681
11	5.50	1.020	153.9	49:30	**1695**	4.4	12,483
12	6.66	1.006	35.6	53:00	**1141**	3.6	4116
13	5.70	1.017	137.0	59:30	**2354**	3.3	5116
14	5.39	1.022	145.9	68:30	**2791**	0.0	1038
15	6.21	1.005	33.9	71:00	**544**	2.2	168
16	5.60	1.018	158.5	79:00	**2957**	11.2	11,046
17	5.68	1.016	116.0	84:30	**2615**	2.1	4669
18	5.55	1.027	240.3	92:30	**3716**	1.3	3312

* Samples were collected from the initial dose until the seventh day following the administration of the last (seventh) dose of clobenzorex. The last sample collected for each subject was at 310:39, 312:30, 301:25, 336:15, and 344:00 hours for subjects 1–5, respectively. Data from samples collected after the last sample that contained detectable amounts of analyte are not included in this table. Bolding indicates positive result for amphetamines.

† Abbreviations: Sp. gr., specific gravity; Amp, amphetamine; Clob, clobenzorex; 4-HO-clob, 4-hydroxyclobenzorex.

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Table I (continued). Analytical Results Following Multidose Administration of Clobenzorex*

Sample	pH	Sp. gr.†	Creatinine	Hours post-first dose	Concentration (ng/mL) Amp	Clob	4-HO-clob
19	6.11	1.022	127.6	100:00	**2310**	7.4	19,713
20	7.28	1.024	203.0	104:00	**2172**	2.8	3696
21	7.08	1.014	102.6	108:30	**1525**	3.7	1439
22	5.68	1.016	151.1	116:00	**2892**	1.6	1062
23	5.80	1.014	109.4	121:30	**1358**	2.3	1414
24	6.41	1.009	55.1	125:30	**2099**	5.3	12,619
25	7.62	1.009	50.6	128:00	495	0.0	1197
26	7.17	1.007	34.3	131:00	**676**	0.0	486
27	6.46	1.008	47.6	133:00	**1275**	1.2	576
28	5.55	1.018	154.0	140:30	**2987**	0.0	1036
29	6.14	1.018	127.8	144:30	**1280**	0.0	563
30	6.38	1.015	85.2	147:30	**2835**	10.9	21,989
31	6.05	1.007	51.7	150:00	**2533**	2.0	1688
32	7.31	1.006	39.1	152:30	**576**	0.0	735
33	6.36	1.017	115.6	156:00	**2373**	0.0	1688
34	5.51	1.010	75.2	159:00	**2908**	0.0	431
35	5.39	1.017	169.9	164:30	**3759**	0.0	1433
36	6.71	1.018	133.6	169:30	**1783**	0.0	840
37	6.94	1.010	60.2	172:30	**570**	0.0	216
38	6.04	1.011	71.1	175:00	107	0.0	221
39	6.74	1.010	53.7	179:30	212	0.0	123
40	5.66	1.020	142.8	188:30	**1230**	0.0	244
41	5.59	1.010	57.1	193:00	335	0.0	73
42	5.23	1.020	70.0	196:00	306	0.0	55
43	6.19	1.022	146.7	201:30	238	0.0	93
44	5.87	1.016	93.9	206:30	250	0.0	48
45	5.95	1.023	169.9	213:30	236	0.0	103
46	6.18	1.007	32.4	216:30	77	0.0	Detected
47	6.16	1.010	52.7	221:30	66	0.0	Detected
48	5.82	1.016	95.5	224:00	91	0.0	Detected
49	5.47	1.023	135.4	230:00	100	0.0	Detected
50	5.51	1.008	44.8	233:30	44	0.0	0
51	5.33	1.018	119.5	240:00	70	0.0	Detected
52	6.84	1.021	129.2	246:30	20	0.0	Detected
53	6.84	1.007	37.9	248:30	0	0.0	0
54	7.40	1.021	131.5	252:00	0	0.0	Detected
55	6.30	1.014	94.8	260:30	17	0.0	0
56	5.54	1.012	69.1	266:30	13	0.0	0
57	6.70	1.015	91.0	271:00	11	0.0	0
58	7.23	1.013	95.4	275:00	0	0.0	0
59	6.41	1.014	95.9	281:30	0	0.0	0
60	6.53	1.011	77.7	284:30	0	0.0	0
61	5.53	1.015	91.5	289:00	11	0.0	0
62	5.30	1.019	136.3	292:30	11	0.0	Detected
63	6.89	1.022	146.6	296:00	7	0.0	Detected
64	7.14	1.006	27.3	299:00	0	0.0	0
65	6.70	1.017	99.0	308:30	5	0.0	0
66	5.28	1.010	62.6	312:30	7	0.0	0
Subject 3							
Pre - 1	5.70	1.007	94.0	00:00	0	0.0	0
2	5.85	1.016	178.0	03:15	**2302**	24.0	13,437
3	6.79	1.003	27.8	03:50	**847**	0.0	557
4	5.89	1.005	51.2	05:20	**1439**	0.0	745
5	5.48	1.007	86.5	07:15	**1918**	0.0	238
6	5.41	1.016	165.0	07:50	**2510**	2.7	718
7	5.62	1.007	70.5	10:05	**1078**	0.0	224
8	5.71	1.010	111.0	13:25	**1029**	0.0	216
9	5.93	1.004	41.1	14:10	45	0.0	68
10	5.99	1.014	154.0	23:30	**822**	0.0	215
11	6.93	1.017	186.0	25:15	**1775**	18.3	16,095
12	6.95	1.004	31.2	26:45	**719**	2.2	2182
13	6.30	1.006	70.1	27:10	**697**	0.0	203
14	7.18	1.003	17.7	27:45	**559**	12.2	423
15	6.34	1.007	54.5	29:35	**1221**	0.0	478
16	6.31	1.015	119.0	30:45	**1444**	0.0	495
17	6.02	1.009	84.0	32:45	**1440**	0.0	281
18	5.71	1.009	83.0	35:15	**1083**	0.0	471
19	5.64	1.010	109.0	47:48	**983**	0.0	193
20	6.26	1.008	69.6	49:05	**782**	0.0	261
21	6.22	1.017	208.0	50:15	**2632**	33.9	17,786
22	5.61	1.011	110.0	53:35	**2578**	4.1	1438
23	5.43	1.012	114.0	54:54	**3364**	2.0	308
24	5.57	1.011	107.0	57:30	**2136**	1.4	616
25	6.34	1.004	19.3	62:00	137	0.0	49
26	6.39	1.012	94.5	71:37	**744**	0.0	3185
27	7.05	1.012	106.0	72:55	**813**	17.9	128
28	6.42	1.004	28.2	73:45	**1350**	4.4	5800
29	6.67	1.005	30.6	75:30	**1114**	1.1	641
30	5.86	1.006	55.8	77:30	**1764**	0.0	411
31	5.74	1.007	67.3	79:05	**1895**	0.0	572
32	5.91	1.016	131.0	80:45	**1862**	0.0	563
33	6.38	1.016	109.0	83:00	**1118**	0.0	298
34	6.33	1.006	47.1	85:30	**555**	0.0	136
35	6.71	1.005	34.3	86:30	433	0.0	68
36	5.84	1.016	187.0	95:05	**1194**	0.0	283
37	5.52	1.017	155.0	99:05	**3311**	22.6	5436
38	5.37	1.017	169.0	101:05	**3588**	4.6	962
39	5.31	1.016	170.0	103:05	**3917**	2.6	1378
40	5.86	1.006	51.9	103:30	**1267**	0.0	273
41	6.45	1.002	17.4	103:55	**518**	0.0	68
42	6.03	1.006	43.3	105:35	**938**	0.0	152
43	7.25	1.015	147.0	107:15	**544**	0.0	396
44	7.27	1.010	67.6	108:30	393	0.0	154
45	6.38	1.004	27.1	110:15	145	0.0	56
46	6.27	1.003	30.2	110:45	436	0.0	55
47	5.81	1.009	88.2	118:45	**827**	0.0	90
48	6.17	1.005	59.4	121:55	**2173**	8.4	1852
49	5.73	1.008	73.4	123:55	**2038**	3.3	684
50	5.90	1.026	318.6	125:00	116	0.0	360
51	5.96	1.005	49.9	125:35	**1209**	0.0	531
52	6.46	1.011	108.7	126:55	**1202**	0.0	763
53	5.60	1.020	198.0	129:45	**2234**	1.5	185
54	5.83	1.009	78.5	133:45	**1196**	0.0	135
55	5.97	1.012	139.0	135:15	**2959**	14.8	7363

* Samples were collected from the initial dose until the seventh day following the administration of the last (seventh) dose of clobenzorex. The last sample collected for each subject was at 310:39, 312:30, 301:25, 336:15, and 344:00 hours for subjects 1–5, respectively. Data from samples collected after the last sample that contained detectable amounts of analyte are not included in this table. Bolding indicates positive result for amphetamines.

† Abbreviations: Sp. gr., specific gravity; Amp, amphetamine; Clob, clobenzorex; 4-HO-clob, 4-hydroxyclobenzorex.

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Table I (continued). Analytical Results Following Multidose Administration of Clobenzorex*

Sample	pH	Sp. gr.†	Creatinine	Hours post-first dose	Concentration (ng/mL) Amp	Clob	4-HO-clob
56	6.09	1.013	131.0	143:37	**925**	0.0	709
57	5.47	1.012	137.0	148:15	**3527**	6.8	991
58	5.60	1.023	239.0	151:15	**3806**	3.0	1546
59	5.49	1.024	290.0	152:50	**4500**	3.4	1193
60	5.44	1.023	235.0	155:35	**3688**	1.7	1016
61	5.46	1.022	204.0	157:15	**3507**	0.0	235
62	5.59	1.014	149.0	167:15	**1250**	0.0	72
63	5.70	1.013	117.0	175:00	352	0.0	63
64	5.79	1.012	116.7	176:45	221	0.0	52
65	5.44	1.028	355.2	182:35	**753**	0.0	378
66	5.32	1.018	174.6	191:00	405	0.0	41
67	5.49	1.024	278.0	197:00	**724**	0.0	266
68	5.60	1.007	56.9	199:15	71	0.0	Detected
69	5.96	1.024	285.6	204:15	81	0.0	40
70	5.26	1.026	272.0	206:45	153	0.0	48
71	5.48	1.011	106.0	215:15	45	0.0	0
72	6.61	1.019	224.0	218:45	17	0.0	Detected
73	7.48	1.010	74.8	219:30	0	0.0	0
74	6.43	1.013	145.9	222:45	16	0.0	Detected
75	6.26	1.018	201.3	225:00	16	0.0	Detected
76	7.00	1.007	68.2	227:30	0	0.0	0
77	5.64	1.007	72.3	229:15	16	0.0	0
78	7.31	1.005	45.1	230:15	0	0.0	0
79	5.81	1.013	141.6	239:45	17	0.0	0
80	7.44	1.016	160.3	241:45	0	0.0	0
81	8.02	1.018	156.8	242:45	0	0.0	0
82	6.07	1.008	78.8	247:45	0	0.0	0
83	6.93	1.020	181.2	250:35	0	0.0	0
84	6.44	1.023	206.5	251:45	0	0.0	0
85	5.77	1.021	164.9	254:15	10	0.0	0
86	5.97	1.010	79.6	261:45	0	0.0	0
87	5.72	1.012	87.5	263:25	0	0.0	0
88	5.77	1.012	91.4	266:15	0	0.0	0
89	6.00	1.007	47.8	267:30	0	0.0	0
90	5.54	1.013	94.8	269:00	0	0.0	0
91	6.72	1.005	24.6	270:45	0	0.0	0
92	6.09	1.016	139.4	275:45	0	0.0	Detected
93	6.26	1.008	52.6	278:00	0	0.0	0
94	5.76	1.010	69.4	283:50	0	0.0	0
95	7.70	1.010	62.1	286:15	0	0.0	0
96	5.74	1.014	99.7	289:15	0	0.0	0
97	6.29	1.007	41.7	290:30	0	0.0	0
98	5.51	1.020	173.9	292:30	12	0.0	Detected
Subject 4							
Pre - 1	5.84	1.025	190.1	00:00	0	0.0	0
2	6.32	1.013	108.1	03:40	**885**	5.6	17,191
3	6.72	1.007	48.5	06:11	**627**	0.0	2286
4	6.94	1.010	73.7	09:57	395	0.0	597
5	6.42	1.014	109.1	12:10	**797**	0.0	425
6	5.33	1.021	195.1	14:32	**2122**	0.0	525
7	6.35	1.016	149.6	15:45	425	0.0	263
8	5.60	1.023	229.4	23:40	**1496**	0.0	231
9	5.49	1.013	109.8	26:05	**889**	1.9	4559
10	6.07	1.015	77.8	28:10	**1617**	5.4	31,586
11	6.60	1.009	47.4	29:34	**653**	0.0	1629
12	5.67	1.015	106.0	36:09	**1519**	0.0	1094
13	5.27	1.011	106.3	39:10	**1713**	0.0	999
14	5.87	1.016	141.3	47:50	**917**	0.0	421
15	5.62	1.023	162.9	51:47	**2397**	5.2	15,279
16	6.84	1.015	73.8	53:51	**1051**	0.0	1387
17	6.88	1.005	29.7	55:13	377	0.0	315
18	6.94	1.002	10.3	55:40	177	0.0	121
19	6.59	1.002	15.2	56:18	274	0.0	164
20	6.24	1.003	27.1	57:00	424	0.0	178
21	5.91	1.007	45.8	58:55	**707**	0.0	320
22	5.90	1.004	20.9	59:48	378	0.0	93
23	5.27	1.013	100.2	62:53	**1392**	0.0	616
24	5.48	1.015	166.7	70:20	**1213**	0.0	407
25	5.57	1.025	254.3	74:12	**1057**	0.0	480
26	6.79	1.020	146.4	77:11	**523**	4.1	34,440
27	6.11	1.020	172.9	84:30	**1629**	4.3	7244
28	5.88	1.027	221.0	98:10	**1813**	0.0	976
29	5.74	1.021	125.0	101:00	**711**	0.0	262
30	5.82	1.020	106.8	102:21	395	0.0	119
31	5.52	1.024	161.6	105:00	**539**	0.0	156
32	7.02	1.028	177.5	109:58	**592**	0.0	5439
33	6.53	1.009	62.0	115:30	**842**	0.0	6837
34	6.55	1.023	248.1	119:50	**3710**	8.3	5418
35	5.35	1.026	267.5	120:51	**3328**	7.0	13,052
36	5.92	1.010	61.6	124:05	**1806**	3.3	15,121
37	5.52	1.017	162.2	128:47	**3083**	4.6	1362
38	5.95	1.021	200.4	130:57	**1549**	0.0	2012
39	5.47	1.024	217.2	133:03	**3165**	0.0	1512
40	5.48	1.013	105.5	134:40	**2581**	0.0	581
41	5.79	1.029	289.2	144:05	**1662**	0.0	1153
42	5.70	1.021	131.7	148:55	**1885**	5.4	26,277
43	5.96	1.023	163.6	150:55	**1096**	4.6	5317
44	6.42	1.026	203.4	156:02	**1096**	0.0	1542
45	5.71	1.025	202.4	156:44	**1406**	0.0	1432
46	5.76	1.028	237.0	162:45	**2442**	0.0	1443
47	5.31	1.033	403.9	168:10	**4275**	0.0	876
48	5.90	1.024	172.4	171:22	**1033**	0.0	555
49	7.21	1.024	177.7	173:50	123	0.0	268
50	6.30	1.025	185.4	180:34	429	0.0	90
51	5.55	1.031	331.7	188:00	**835**	0.0	105
52	5.51	1.028	263.8	192:50	**552**	0.0	48
53	5.42	1.026	247.3	196:00	390	0.0	48
54	6.89	1.024	152.7	202:56	54	0.0	Detected
55	5.51	1.027	194.5	207:30	206	0.0	Detected
56	5.53	>1.035	292.4	216:20	239	0.0	32
57	5.36	1.032	293.1	219:46	157	0.0	Detected
58	6.32	1.024	148.5	223:05	22	0.0	0
59	6.62	1.023	122.4	228:30	15	0.0	0
60	5.45	1.027	178.8	231:49	42	0.0	Detected

* Samples were collected from the initial dose until the seventh day following the administration of the last (seventh) dose of clobenzorex. The last sample collected for each subject was at 310:39, 312:30, 301:25, 336:15, and 344:00 hours for subjects 1–5, respectively. Data from samples collected after the last sample that contained detectable amounts of analyte are not included in this table. Bolding indicates positive result for amphetamines.

† Abbreviations: Sp. gr., specific gravity; Amp, amphetamine; Clob, clobenzorex; 4-HO-clob, 4-hydroxyclobenzorex.

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Table I (continued). Analytical Results Following Multidose Administration of Clobenzorex*

Sample	pH	Sp. gr.†	Creatinine	Hours post-first dose	Concentration (ng/mL) Amp	Clob	4-HO-clob
61	5.61	1.030	248.0	244:00	34	0.0	Detected
62	5.39	1.022	155.6	247:24	16	0.0	Detected
63	5.74	1.025	168.1	255:15	14	0.0	Detected
64	5.35	1.031	390.8	265:00	34	0.0	30
65	7.56	1.025	127.4	269:30	0	0.0	0
66	7.28	1.027	190.5	273:30	0	0.0	0
67	5.87	1.023	171.7	275:00	8	0.0	0
68	5.71	1.026	239.6	279:13	0	0.0	Detected
69	5.59	1.022	227.1	286:30	9	0.0	0
Subject 5							
Pre - 1	5.34	1.029	330.2	00:00	0	0.0	0
2	6.32	1.023	181.5	01:30	104	4.0	25,510
3	6.71	1.012	87.3	04:25	383	0.0	4952
4	6.77	1.022	186.9	07:00	374	0.0	3528
5	5.90	1.026	273.4	12:45	**1017**	0.0	3010
6	5.48	1.028	222.2	18:00	**1250**	0.0	738
7	5.45	1.016	129.1	22:10	**617**	0.0	351
8	5.68	1.022	183.5	25:25	376	0.0	334
9	5.77	1.023	145.4	30:10	**681**	7.7	28,928
10	5.53	1.027	276.1	37:20	**1781**	7.3	8683
11	5.39	1.032	396.8	46:15	**1665**	0.0	1587
12	6.42	1.027	260.2	51:10	**769**	6.3	37,862
13	5.96	1.016	124.4	60:30	**1008**	0.0	1063
14	5.46	1.024	270.2	61:00	**2036**	0.0	1316
15	5.35	1.024	257.4	70:30	**1490**	0.0	929
16	6.36	1.026	227.9	75:35	440	0.0	526
17	5.52	1.016	132.4	81:30	**1751**	2.6	2915
18	5.91	1.010	72.6	83:30	**767**	0.0	616
19	6.06	1.005	38.8	85:00	**411**	0.0	470
20	5.42	1.021	247.0	90:00	**1887**	0.0	1343
21	5.57	1.024	221.0	97:00	**1081**	0.0	457
22	6.21	1.024	227.9	103:40	**1566**	10.3	53,905
23	6.17	1.023	173.4	105:15	276	0.0	162
24	5.73	1.025	187.2	107:45	369	0.0	110
25	5.89	1.021	152.0	110:00	264	0.0	82
26	6.04	1.013	76.9	111:30	244	2.6	17,048
27	5.54	1.023	224.4	113:40	**2767**	46.6	69,837
28	5.72	1.026	256.7	117:40	**2478**	8.8	5687
29	6.09	1.031	315.4	121:50	**1370**	0.0	823
30	6.60	1.022	218.0	122:00	**712**	0.0	1516
31	6.25	1.025	299.6	127:30	**1886**	17.3	99,044
32	5.82	1.024	180.3	129:35	**1290**	0.0	3019
33	5.59	1.025	249.5	132:00	**2850**	6.1	5312
34	5.75	1.031	391.3	141:50	**2891**	0.0	1283
35	6.96	1.021	167.7	147:15	**629**	10.2	49,246
36	6.10	1.024	227.0	150:10	**1591**	8.0	18,063
37	5.65	1.024	197.8	151:40	**1905**	2.3	3083
38	6.15	1.022	158.5	156:00	**800**	0.0	1525
39	6.36	1.021	149.9	157:40	**503**	0.0	1136
40	6.13	1.021	147.7	158:40	**646**	0.0	748
41	5.89	1.026	280.8	159:30	**1583**	0.0	2461
42	5.86	1.019	125.1	164:30	**800**	0.0	334
43	6.02	1.024	167.3	169:35	**563**	0.0	355
44	5.86	1.026	243.2	173:15	475	0.0	171
45	5.92	1.027	215.0	175:05	343	0.0	82
46	5.89	1.027	204.3	180:30	284	0.0	89
47	5.66	1.028	231.7	182:30	340	0.0	79
48	5.56	1.027	188.8	189:45	288	0.0	47
49	5.70	1.027	180.9	193:15	165	0.0	40
50	5.76	1.025	176.8	195:20	110	0.0	28
51	5.40	1.028	228.2	199:30	139	0.0	25
52	5.89	1.026	196.7	201:15	71	0.0	Detected
53	5.49	1.027	193.1	206:00	88	0.0	Detected
54	5.55	1.024	218.0	214:40	80	0.0	30
55	5.33	1.028	244.0	221:00	38	0.0	Detected
56	5.76	1.027	213.9	222:55	25	0.0	Detected
57	5.69	1.029	353.2	230:50	30	0.0	Detected
58	5.46	1.029	429.4	237:30	38	0.0	Detected
59	5.36	1.027	287.2	245:10	14	0.0	Detected
60	5.50	1.029	297.9	253:00	12	0.0	Detected
61	5.79	1.025	189.1	261:30	0	0.0	0
62	5.58	1.010	83.0	266:30	0	0.0	0
63	5.31	1.025	213.0	272:30	5	0.0	0
64	5.31	1.023	195.8	277:00	0	0.0	0
65	6.26	1.021	172.8	280:15	0	0.0	0
66	5.59	1.026	283.9	289:10	0	0.0	Detected
67	5.63	1.027	274.2	295:10	0	0.0	0
68	5.39	1.031	414.3	300:30	7	0.0	0

* Samples were collected from the initial dose until the seventh day following the administration of the last (seventh) dose of clobenzorex. The last sample collected for each subject was at 310:39, 312:30, 301:25, 336:15, and 344:00 hours for subjects 1–5, respectively. Data from samples collected after the last sample that contained detectable amounts of analyte are not included in this table. Bolding indicates positive result for amphetamines.
† Abbreviations: Sp. gr., specific gravity; Amp, amphetamine; Clob, clobenzorex; 4-HO-clob, 4-hydroxyclobenzorex.

Results and Discussion

Following administration of clobenzorex to five subjects, amphetamine, clobenzorex, and 4-HO-clobenzorex were detected in the urine of all five. In previous studies (1–3), amphetamine and the parent drug clobenzorex were found in the urine, but clobenzorex was not detected in all samples positive for amphetamine. Initial work by Maurer et al. (3) identified several metabolites of clobenzorex, including two different isomers of hydroxyclobenzorex, one of which was reportedly detected as long as amphetamine was in the urine of at least one of the subjects. In another report, 4-HO-clobenzorex was measured in samples from a study involving the administration of a single 30-mg dose of clobenzorex to five subjects. In that study, peak analyte concentrations were amphetamine 715–2474 ng/mL, clobenzorex 10–29 ng/mL, and 4-HO-clobenzorex 5705–88,410 ng/mL. Total excretion of the drug and metabolites showed approximately 6–15% of the dose was excreted as amphetamine, 10–40% as 4-HO-clobenzorex, and less than 1% as the unchanged parent drug (4).

The results of sample analysis from this study are given in Table I. For each subject, changes in the urine concentration of

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the analytes corresponded not only with time since dose, but also with changes in pH or creatinine and specific gravity consistent with the expected effects these parameters represent with respect to dilution and changes in ionization of these amine containing compounds (see Table I).

When present, the parent drug is a valuable indicator of the use of this drug. In this case, identification of the parent drug has the distinct advantage of using the same analytical procedure employed to identify amphetamine with the simple additional step of monitoring appropriate ions for the drug (1). Use of this procedure is the most practical approach for most laboratories unless clobenzorex is not detected and there is a question regarding the involvement of this drug, in which case the hydroxy metabolite would be the method of choice. To detect 4-HO-clobenzorex, the sample must be hydrolyzed, a procedure not used in many drug-testing laboratories. Unfortunately, many amphetamine-positive samples do not contain the parent drug. In one subject, as many as 70% of the amphetamine-positive samples contained no detectable clobenzorex, whereas all contained the 4-HO-clobenzorex metabolite. Therefore, identification of the hydroxy metabolite is clearly a better indicator to determine use of this drug in amphetamine-positive samples.

Following hydrolysis, numerous other compounds are extracted from the sample compared to samples that are not hydrolyzed. In a few samples, typically those collected shortly after administration of the drug, a compound eluted close to clobenzorex and its internal standard. In those instances, use of the ZB-50 rather than the HP-1 column typically eliminated the interference. Dilution of the sample also minimized the impact of the interfering compounds. Many of these samples required dilution to bring the concentration of 4-HO-clobenzorex within the linear range. Although it was possible to eliminate interference with the parent compound in almost all cases, the potential for interference may compromise the quantitative accuracy of the clobenzorex when using this method.

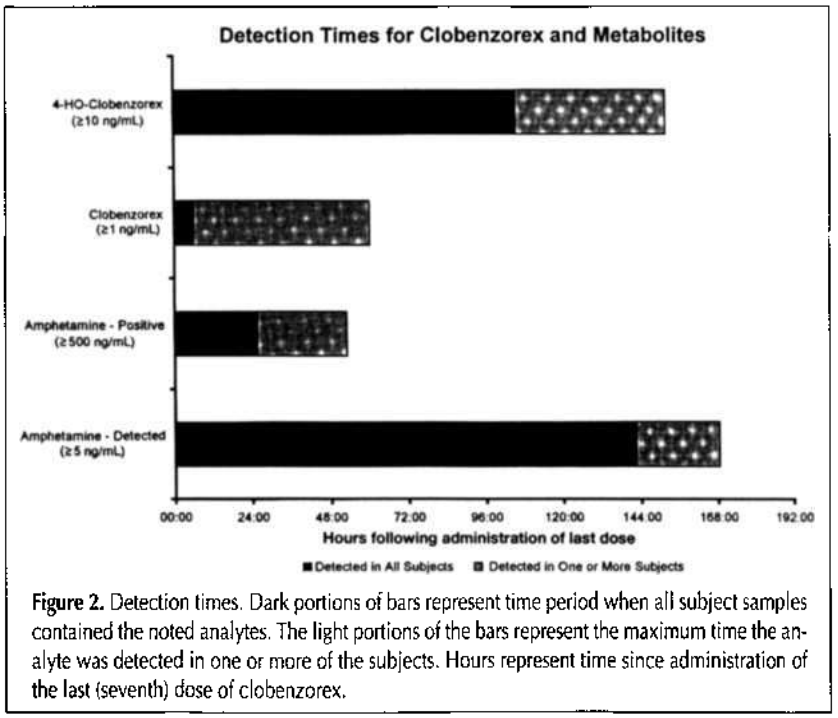

Figure 2. Detection times. Dark portions of bars represent time period when all subject samples contained the noted analytes. The light portions of the bars represent the maximum time the analyte was detected in one or more of the subjects. Hours represent time since administration of the last (seventh) dose of clobenzorex.

Amphetamine was first detected (LOD = 5 ng/mL) from 1:30 to 4:00 hours post-first-dose. The first amphetamine positive (≥ 500 ng/mL) was seen at 3:15–12:45 hours post-first-dose and the last positive at 25:35–53:00 hours post-last-dose. Peak concentrations of amphetamine were 2891–4716 ng/mL. Amphetamine was last detected from 142:30 to 168:30 hours post-last-dose. The first detectable level of clobenzorex (LOD = 1 ng/mL) ranged from 1:30 to 4:00 hours post-first-dose for all five subjects with peak concentrations ranging from 8 to 47 ng/mL. It was last detected from 6:00 to 60:10 hours post-last-dose. 4-Hydroxyclobenzorex was first detected (LOD = 10 ng/mL) from 1:30 to 4:00 hours post-first-dose with peak concentrations of 17,786–99,044 ng/mL. It was last detected from 105:35 to 152:00 post-last-dose (See Figure 2 for summary of detection times for each analyte).

Although peak concentrations of 4-HO-clobenzorex far exceeded those of amphetamine, it was also excreted more rapidly than was amphetamine. Following a single dose of clobenzorex, amphetamine was detected in samples after 4-HO-clobenzorex could no longer be found in four of five subjects. In the fifth subject, the last detectable amounts of both compounds were in the same sample. The 4-HO-clobenzorex was seen at higher concentrations for the first 10–32 h postdose, with peak concentrations ranging from 2.3 to 51 times that of amphetamine. After this point, a crossover occurred where amphetamine was most often found in greater amounts than 4-HO-clobenzorex, although at low concentrations (< 100 ng/mL), the concentration of 4-HO-clobenzorex sometimes again exceeded that of amphetamine. In every case, however, high concentrations of 4-HO-clobenzorex were indicative of recent use (4).

In the current multidose study, the peak concentration of amphetamine reached 4716 ng/mL, compared to 2473 ng/mL following a single dose. The increased concentration is reasonably expected following administration of multiple doses. Review of Table I shows the increased concentrations were evident within the first few days, and it appears unlikely that use of the drug at the same dosage level for an extended period of time would lead to significantly higher concentrations of amphetamine. The 4-hydroxy metabolite was found at slightly higher peak concentrations than seen in the single-dose study. Consistent with the findings of the single-dose study, there was a corresponding sharp decline in concentration before administration of the next dose of the drug. In this study, the crossover observed in the single dose study is not as obvious; however, in all cases the amount of 4-HO-clobenzorex dropped to nearly the same or lower concentration than amphetamine within the 24 h following administration of the dose. Examination of Table I shows the amphetamine concentration relative to the concentration of 4-HO-clobenzorex can be very valuable in evaluating time since dose.

Interpretation of drug testing results is sometimes challenging but can be helped by knowing as much about the sample as pos-

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sible. Enantiomeric composition and evaluation of quantitative data can provide significant help in that endeavor. The fact that clobenzorex is metabolized to *d*-amphetamine only (2) is a valuable tool for interpretation of results. By itself, enantiomeric composition can not establish the use of this drug. Rather, the presence of the *l*-enantiomer is inconsistent with the use of clobenzorex. Although the amphetamine concentrations seen in this study exceed those seen in the single-dose study, they are much lower than levels seen in many amphetamine abuse cases, which can also help differentiate the source of the amphetamine. In the interpretation of analytical data, differentiating amphetamine use from clobenzorex use in samples with a low level of amphetamine would be difficult; however, high concentrations of amphetamine would be inconsistent with the ordinary therapeutic use of this drug. Because urine samples were not pooled over time, and no attempt was made to control urine pH or degree of hydration, the samples represent a wide distribution of pH, specific gravity, and creatinine values as would be expected in routine samples submitted to a urine drug-testing laboratory (see Table I). Another important finding of the current study is that all samples containing amphetamine at or above the 500-ng/mL cutoff showed detectable amounts of 4-HO-clobenzorex.

Conclusions

As seen in a previously reported single-dose study (4), administration of clobenzorex resulted in urinary excretion of amphetamine and variable but small amounts of the parent drug. Although clobenzorex was detected in many of the urine samples for hours following administration, it was not detected in all amphetamine-positive samples (≥ 500 ng/mL). However, the metabolite 4-HO-clobenzorex was found in all amphetamine-positive samples. The metabolite, 4-hydroxyclobenzorex proved to be an excellent indicator of clobenzorex use compared to the parent drug. Data presented here can help differentiate between use of clobenzorex and amphetamine abuse.

Acknowledgments

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The voluntary, fully informed consent of the subjects used in this research was obtained as required by AFI 40-403.

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Defense or other Departments of the U.S. Government.

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Differentiation of Clobenzorex Use from Amphetamine Abuse Using the Metabolite 4-Hydroxyclobenzorex*,†

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Abstract

Clobenzorex (Asenlix) is an anorectic drug metabolized by the body to amphetamine, thus causing difficulty in the interpretation of amphetamine-positive drug tests. Previous studies have shown the parent drug and several metabolites are excreted in urine. Clobenzorex itself has been detected for as long as 29 h postdose using a detection limit of 1 ng/mL. Despite this fact, several amphetamine-positive samples (≥ 500 ng/mL) contained no detectable clobenzorex. Thus, the absence of clobenzorex in the urine does not exclude the possibility of its use. To more definitively assess the possibility of clobenzorex use, evaluation of another metabolite was considered. One study reported the presence of unidentified hydroxy metabolites of clobenzorex for as long as amphetamine was detected in some subjects. To assess the viability of using a hydroxy metabolite to confirm the use of clobenzorex in samples containing amphetamine, 4-hydroxyclobenzorex was synthesized for this study. This metabolite proved to be easily detected and was typically found at levels higher than amphetamine in amphetamine-positive urines, long after clobenzorex itself was no longer detected. Samples obtained from a controlled single-dose study involving the administration of clobenzorex (30 mg) were analyzed for the presence of the 4-hydroxy metabolite. The analytical procedure used acid hydrolysis followed by liquid–liquid extraction and analysis with gas chromatography–mass spectrometry by monitoring ions at *m/z* 125, 330, and 364. 4-Hydroxyclobenzorex and its 3-Cl regioisomer were used in the identification and quantitation of the metabolite. Peak concentrations of 4-hydroxyclobenzorex were found at approximately 1:30–5:00 h postdose and ranged from approximately 5705 to 88,410 ng/mL. Most importantly, however, all samples that contained amphetamine at ≥ 500 ng/mL also contained detectable amounts of this hydroxy metabolite (LOD 10 ng/mL), making it a valuable tool in differentiating use of clobenzorex from illicit amphetamine use.

* This work was supported in part by a Lucas Research Grant and USAF SGO funds I95-228I. The voluntary, fully informed consent of the subjects used in this research was obtained as required by AFI 40-403.

† The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Defense or other Departments of the U.S. Government.

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Introduction

Clobenzorex, N[(2-chlorophenyl)methyl]-α-methyl-benzeneethanamine, is an anorectic drug that is not sold in the United States but is available in many countries as a prescription (e.g., in Mexico) or over-the-counter (e.g., in Panama) drug. The recommended starting dose is 30 mg (one capsule) per day. It is one of several so-called "precursor drugs" that are metabolized to amphetamine by the body and excreted in the urine. Because it is metabolized to amphetamine, therapeutic use of clobenzorex may lead to suspicion of amphetamine abuse. Drugs that are metabolized to amphetamine and/or methamphetamine are potentially significant concerns in the interpretation of amphetamine-positive drug-testing results. The presence of the parent compound or a unique metabolite can be conclusive evidence for the involvement of this drug.

Synthesis of a regioisomer of clobenzorex for use as internal standard and use of selected ion monitoring gas chromatography–mass spectrometry (GC–MS) led to an accurate quantitative method with the limit of detection (LOD) and limit of quantitation (LOQ) for clobenzorex at 1 ng/mL. However, even with this low LOD, the parent compound was not always detected in samples positive for amphetamine (≥ 500 ng/mL, the Health and Human Services and Department of Defense administrative cutoff level for amphetamine in urine), and when detected, it was most often found at very low concentrations (1). In view of this fact, it appeared that evaluation of a metabolite might prove to be a better indicator of clobenzorex use. Several metabolites, including two different hydroxy metabolites, have previously been identified (2). Both were detected longer than the parent drug, with one detectable for approximately the same amount of time as amphetamine.

Because it seemed most likely that hydroxylation would take place at the 4 position on the ring, 4-hydroxyclobenzorex (4-HO-clobenzorex) and its regioisomer, *N*-3-chlorobenzyl-4-hydroxyamphetamine (3-Cl-4-HO-clobenzorex), were synthesized for use in this study (see Figure 1 for structures). Because hydroxy metabolites are typically excreted as conjugates, hydrolysis was performed prior to extraction and derivatization.

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Hydrolysis methods (enzyme vs. acid) were evaluated as part of this study.

Materials and Methods

Materials

Amphetamine, methamphetamine, amphetamine-d_6 (1-phenyl-2-aminopropane-1,1,2,3,3,3-d_6), and methamphetamine-d_{11} (1-phenyl-d_5-2-methyl-d_3-aminopropane-3,3,3-d_3) were obtained from Radian Corp. Clobenzorex hydrochloride was obtained through colleagues in Spain. The internal standard, 3-chlorobenzylamphetamine (3-Cl-clobenzorex), was synthesized in our laboratory following the procedure described by Lintermans et al. (3). The standard, 4-hydroxyclobenzorex (4-HO-clobenzorex), and the internal standard, *N*-3-chlorobenzyl-4-hydroxyamphetamine (3-Cl-4-HO-clobenzorex), were obtained from Isotec, Inc. Heptafluorobutyric anhydride (HFBA) and β-glucuronidase from *Helix pomatia* were obtained from Sigma Chemical Co. Clobenzorex administered to experimental subjects in the form of Asenlix was purchased from a pharmacy in Mexico.

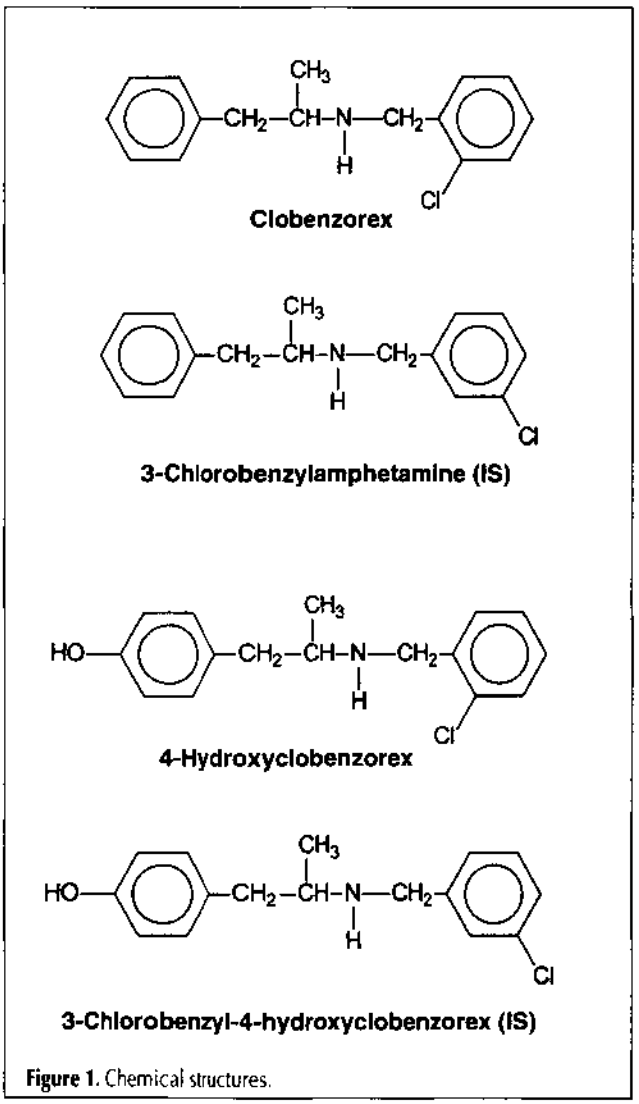

Figure 1. Chemical structures.

Drug administration and sample collection

Thirty milligrams of clobenzorex, in the form of a single Asenlix capsule (Roussel, Mexico), was administered orally to five healthy volunteers (two female and three male) with no history of amphetamine, methamphetamine, or clobenzorex use. A predose sample was taken prior to drug administration, and following administration, urine was collected for the next seven

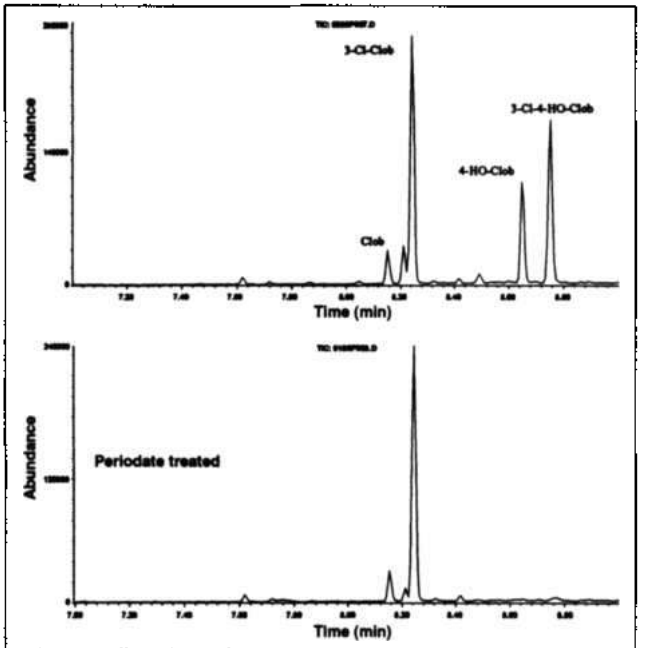

Figure 2. Effect of periodate.

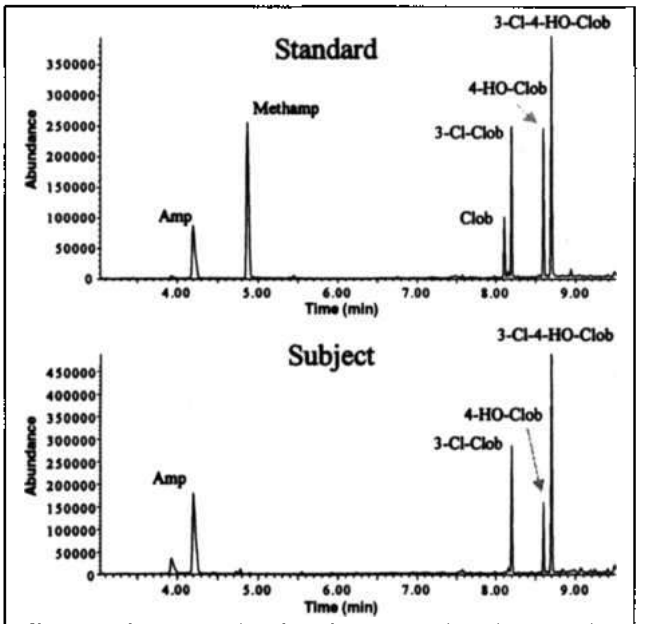

Figure 3. Chromatography of amphetamine and amphetamine-d_6, methamphetamine and methamphetamine-d_{11}, clobenzorex, 3-Cl-clobenzorex, 4-HO-clobenzorex, and 3-Cl-4-HO-clobenzorex from calibration standard containing 500 ng/mL amphetamine, methamphetamine, and 4-HO-clobenzorex; 25 ng/mL clobenzorex; 250 ng/mL deuterated internal standards; 500 ng/mL 3-Cl-4-HO-clobenzorex; and 50 ng/mL 3-Cl-clobenzorex (standard). Chromatography of amphetamine, clobenzorex, and 4-HO-clobenzorex from a urine sample collected following use of clobenzorex (subject).

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days. Total void volume was recorded with each sample to facilitate determination of the total amount of drug and metabolite excreted. Samples were provided ad lib and refrigerated or frozen until analysis. No attempt was made to physiologically control urine pH.

Sample preparation and analysis

Sample pH was measured using a Fisher Accumet 50 pH meter and specific gravity determined using an AO Scientific Instruments refractometer. Creatinine levels were determined at the Wilford Hall Medical Center clinical laboratory using standard clinical laboratory procedures. GC–MS analyses were performed using a Hewlett-Packard 5890 II GC coupled with an HP 5971 MS using a 7673 autoinjector.

Hydrolysis methods

Both enzyme and acid hydrolysis were evaluated to determine which method showed the greatest degree of hydrolysis. Enzyme conditions remained constant throughout the evaluation.

Enzyme hydrolysis (4). One milliliter of enzyme in acetate buffer was added to 2 mL of urine (2600 u/mL β-glucuronidase). Samples were mixed, heated to 56°C for 2 h in a waterbath, cooled to room temperature, then centrifuged at 2500 rpm for 5 min. The supernatent was transferred to a clean glass tube and then extracted.

Acid hydrolysis (2). One milliliter of 30% hydrochloric acid was added to 2 mL of urine. Acid hydrolysis of samples was evaluated using a drybath, waterbath, autoclave, and microwave at various conditions: both the waterbath and drybath were used in evaluating samples at 60°C for 15 min, 75°C for 15 min, and 75°C for 30 min. Because results were comparable between the two, only the waterbath was used to evaluate hydrolysis at 90°C for 15 min and 90°C for 30 min. Time periods used in testing acid hydrolysis of samples by autoclave (121°C, 15 psi) were 15, 30, 45, and 60 min. Microwave conditions consisted of samples exposed to low-medium power for 2 and 5 min, medium power for 5 min, and low power for 10 min.

Quantitative analysis

One milliliter of 30% hydrochloric acid was added to 2 mL of urine. Caps were screwed on loosely, samples were mixed, and then placed in an autoclave (15 psi) for 30 min. Samples were allowed to cool to room temperature followed by extraction, derivatization with heptafluorobutyric anhydride (HFBA), and analysis by GC–MS as previously described (5). Addition of sodium periodate prior to extraction is an optional step in this procedure to eliminate interferences from high concentrations of hydroxyphenylisopropylamines, but it negatively impacted the hydroxy metabolite (Figure 2). Conditions for the HP-1 column (12-m × 0.2-mm i.d., 0.33-µm film thickness) consisted of a temperature program with an initial time of 1 min at 80°C, programmed to 180°C at 20°C/min, then to 250°C at 28°C/min with a 1 min final time. Quantitation and detection was based on single-point calibration using a calibration standard containing 500 ng/mL of amphetamine, methamphetamine, and 4-hydroxyclobenzorex with 250 ng/mL of deuterium-labeled isotopomer internal standards for amphetamine and methamphetamine, 500 ng/mL of 3-Cl-4-HO-clobenzorex, and 25 ng/mL clobenzorex with 50 ng/mL of 3-Cl-clobenzorex as internal standard. Detection limits for this assay were 5 ng/mL for amphetamine and methamphetamine, 1 ng/mL for clobenzorex, and 10 ng/mL for 4-HO-clobenzorex. The limit of quantitation for amphetamine and methamphetamine was 5 ng/mL, 1 ng/mL for clobenzorex, and 25 ng/mL for 4-HO-clobenzorex. Ions monitored were as follows: *m/z* 240, 118, and 91 for amphetamine; *m/z* 254, 210, and 118 for methamphetamine; *m/z* 244 and 123 for amphetamine-d_6; *m/z* 260 and 213 for methamphetamine-d_{11}; *m/z* 118, 125, and 364 for clobenzorex and 3-Cl-clobenzorex; and *m/z* 125, 330, and 364 for 4-HO-clobenzorex and 3-Cl-4-HO-clobenzorex.

Twelve samples were analyzed to determine the efficiency of the extraction procedure. Drug and internal standard (4-HO-clobenzorex and 3-Cl-4-HO-clobenzorex) were added to six samples prior to extraction. For the remaining six samples, internal standard was added after the samples had been extracted. The mean and standard deviation were calculated for each set of samples and extraction efficiency determined. The linear range was determined for this procedure by the analysis of at least seven different runs with concentrations ranging from 10 to 10,000 ng/mL 4-HO-clobenzorex. Precision was measured by testing replicates of 4-HO-clobenzorex at 500 ng/mL and internal standard at 500 ng/mL.

Results and Discussion

Ingestion of clobenzorex results in the metabolic production of amphetamine and other metabolites. Clobenzorex was administered to five healthy subjects with no history of amphetamine or clobenzorex use. As expected, methamphetamine was not detected in any of the subject samples (LOD for methamphetamine was 5 ng/mL). In a previous study (1), amphetamine and clobenzorex were detected in the urine of all five subjects. However, clobenzorex was not detected in all positive amphetamine samples, and when detected, was found at very low concentrations (≤ 29 ng/mL). It was also detected for only a short period of time (up to 29:00 h postdose). In view of these findings, analysis for the parent drug did not appear to be a good indicator of clobenzorex use, so evaluation of a metabolite was considered.

A study by Maurer et al. (2) described metabolites found in urine following ingestion of clobenzorex. Although quantitative analyses of these metabolites was not reported, it was apparent that one of these metabolites, a hydroxy metabolite, was shown to be detected in samples for times comparable to amphetamine. Two different isomers of hydroxyclobenzorex were identified. Isomer 2 was detected for 56 to 84 h postdose, whereas other metabolites were seen for only 12 to 24 h. For these reasons, hydroxyclobenzorex was considered to be the best candidate in evaluating a method that would more definitively establish involvement of clobenzorex. The standard, 4-HO-clobenzorex, and its corresponding regioisomer, *N*-3-Cl-4-HO-clobenzorex, were synthesized for use in this study.

Samples obtained from a controlled single-dose study de-

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Table I. Summary of Amphetamine, Clobenzorex, and 4-Hydroxyclobenzorex Detection

Subject	Amp* first detected (≥ 5 ng/mL) (h postdose)	Amp first positive (≥ 500 ng/mL) (h postdose)	Amp last positive (≥ 500 ng/mL) (h postdose)	Amp last detected (≥ 5 ng/mL) (h postdose)	Clob first detected (≥ 1 ng/mL) (h postdose)	Clob last detected (≥ 1 ng/mL) (h postdose)	4-HO-clob first detected (≥ 10 ng/mL) (h postdose)	4-HO-clob last detected (≥ 10 ng/mL) (h postdose)	Maximum concentration Amp (ng/mL)	Maximum concentration Clob (ng/mL)	Maximum concentration 4-HO-clob (ng/mL)
1	4:30	4:30	7:30	116:00	4:30	7:30	1:30	59:45	715	12	22,910
2	2:40	2:40	23:00	95:00	2:40	19:30	2:40	67:00	2474	29	88,410
3	5:00	5:00	35:59	77:30	5:00	5:00	5:00	77:30	2233	22	16,671
4	1:30	7:30	20:30	91:30	1:30	11:30	1:30	45:30	2399	10	5705
5	1:00	3:00	37:00	104:00	3:00	29:00	1:00	91:30	2232	18	27,249

*Abbreviations: Amp, amphetamine; Clob, clobenzorex; and 4-HO-clob, 4-hydroxyclobenzorex.

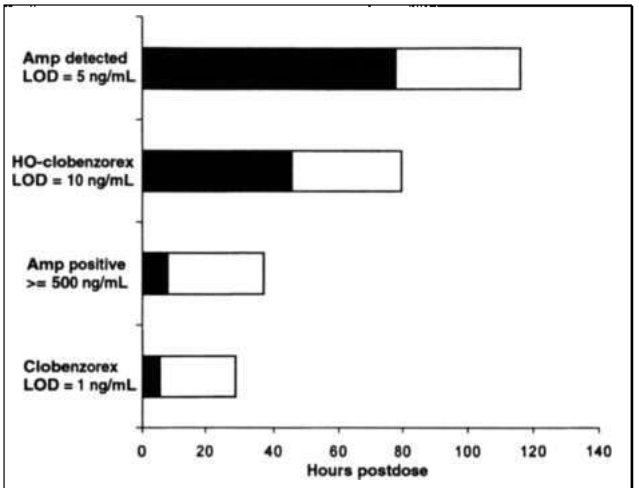

Figure 4. Detection times. The dark portion of bar represents time period when all five subject samples contained the noted analytes. The white portion of the bar represents the maximum time the analyte was detected in any of the subjects.

Table II. Percent of Dose Excreted

Subject	% of Dose excreted as Amp*	% of Dose excreted as Clob	% of Dose excreted as 4-HO-clob
1	6.6	0.01	22.2
2	8.8	0.02	40.4
3	5.9	0.01	10.1
4	15.4	0.02	13.7
5	13.6	0.01	18.5

* Abbreviations: Amp, amphetamine; Clob, clobenzorex; 4-HO-clob, 4-HO-clobenzorex.

scribed earlier were analyzed for the presence of the 4-hydroxy metabolite. Because hydroxy metabolites are typically excreted as conjugates, hydrolysis was performed prior to extraction and derivatization. Both enzyme and acid hydrolysis were evaluated in this study. For enzyme hydrolysis, 1 mL of enzyme (*Helix pomatia*) in acetate buffer was added to 2 mL of urine, and samples were mixed and placed in waterbath at 56°C for 2 h. After incubation, samples were allowed to cool and centrifuged, and then the supernatant was transferred to a clean glass tube. Enzyme conditions remained constant throughout hydrolysis evaluation. Six consecutive runs were analyzed, and although quantitative results were higher, analysis of enzyme hydrolysis showed considerable interference with the peak of interest.

Various conditions were tested using acid hydrolysis. One milliliter of 30% HCl was added to 2 mL of urine. Acid hydrolyzed samples were evaluated using a drybath, waterbath, autoclave, and microwave. Drybath and waterbath sample results were comparable at 60°C for 15 min, 75°C for 15 min, and 75°C for 30 min; therefore, the waterbath was used to evaluate hydrolysis at 90°C for 15 and 30 min. Higher concentrations of drug were seen with higher temperature (90°C); however, autoclaved samples were consistently much higher than samples tested using the waterbath. Samples were autoclaved (121°C, 15 psi) for 15, 30, 45, and 60 min. Results from samples autoclaved for 15 min were lower than results from other autoclave times tested. There was no difference in results from samples autoclaved at 30, 45, or 60 min; therefore, it was determined that samples autoclaved for 30 min provided the best results in the shortest time. Use of a microwave was evaluated to determine if hydrolysis could be accomplished in a shorter period of time. The conditions included microwaving for 2 and 5 min at low-medium power, 5 min at medium power, or 10 min at low power. Results from microwaved samples were all low compared to autoclaved sample results. Samples placed in the autoclave showed the most consistent results. Consequently, the hydrolysis procedure used in this study was that of adding acid to samples, then placing samples in an autoclave for 30 min.

The potential interference from high concentrations of hydroxyphenylisopropylamines (e.g., phenylpropanolamine, ephedrine, etc.) is commonly addressed in amphetamine assays by the use of sodium periodate. To assess the potential effect of periodate on the analysis of 4-HO-clobenzorex, samples were treated with 1 mL of 0.35M sodium periodate at room temperature for 15 min. This procedure effectively eliminated any interference from hydroxyphenylisopropylamines, but also seriously impacted the recovery of 4-HO-clobenzorex and its internal standard. As shown in Figure 2, the 4-hydroxy metabolite and its regioisomer were eliminated when periodate was used as part of the extraction procedure. Therefore, it is important to consider that if periodate is used as part of an amphetamine assay, the possibility of detection of 4-HO-clobenzorex will be diminished or eliminated. Periodate treatment did

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Table III. Analytical Results Following Single-Dose Administration of Clobenzorex*

Subject	Hours post dose	pH	Specific gravity	Concentration (ng/mL) Creatinine	Amp†	Clob‡	4-HO-clob§
1	00:00	5.96	1.005	40	0	0.0	0
1	01:30	7.48	1.007	37	0	0.0	3527
1	04:30	6.69	1.013	112	715	12.1	22,910
1	07:30	6.40	1.008	74	535	3.6	1750
1	08:30	7.21	1.003	22	100	0.0	373
1	10:00	7.33	1.003	21	60	0.0	486
1	12:30	7.14	1.004	36	129	0.0	488
1	14:00	7.36	1.003	17	46	0.0	143
1	17:00	6.89	1.004	31	116	0.0	131
1	20:00	6.13	1.006	54	315	0.0	363
1	23:00	6.27	1.006	63	264	0.0	356
1	25:00	7.10	1.005	35	82	0.0	73
1	26:40	7.21	1.004	35	47	0.0	73
1	29:30	6.96	1.005	38	59	0.0	85
1	33:00	6.23	1.008	79	136	0.0	88
1	35:00	7.03	1.004	31	29	0.0	37
1	37:00	6.69	1.007	70	80	0.0	78
1	41:47	6.58	1.010	92	49	0.0	81
1	47:00	5.92	1.007	57	55	0.0	66
1	48:30	6.07	1.005	36	51	0.0	28
1	50:00	6.73	1.003	14	17	0.0	0
1	51:30	6.78	1.004	28	19	0.0	0
1	55:00	6.47	1.005	40	24	0.0	0
1	56:30	6.38	1.003	28	19	0.0	0
1	59:45	6.52	1.011	100	31	0.0	Detected
1	61:15	6.85	1.005	41	12	0.0	0
1	63:20	6.71	1.002	19	10	0.0	0
1	67:30	5.81	1.005	57	25	0.0	0
1	70:00	5.74	1.008	87	31	0.0	0
1	72:35	6.16	1.004	37	10	0.0	0
1	74:15	6.44	1.004	31	8	0.0	0
1	78:00	6.32	1.004	39	8	0.0	0
1	81:00	6.06	1.006	72	11	0.0	0
1	87:00	5.87	1.024	395	24	0.0	0
1	92:00	6.28	1.008	79	7	0.0	0
1	97:30	5.51	1.007	73	9	0.0	0
1	103:00	5.92	1.012	180	0	0.0	0
1	106:50	5.45	1.009	98	7	0.0	0
1	109:30	5.62	1.014	155	7	0.0	0
1	116:00	5.64	1.024	300	8	0.0	0
2	00:00	6.22	1.022	169	0	0.0	0
2	02:40	6.85	1.023	172	1331	28.8	88,410
2	04:45	6.54	1.021	138	398	2.0	8833
2	06:35	5.60	1.020	194	1721	14.1	3223
2	07:25	6.65	1.009	61	469	0.0	469
2	08:40	6.76	1.011	89	469	0.0	731
2	10:00	5.83	1.007	60	389	0.0	356
2	12:20	5.45	1.021	310	420	0.0	713
2	19:30	5.12	1.023	382	2474	2.3	551
2	23:00	5.26	1.022	370	1187	0.0	258

* Data from samples following the last analyte-containing sample from each subject are not included in the table. None of these samples contained detectable amphetamine (Amp), clobenzorex (Clob), or 4-HO-clobenzorex (4-HO-clob).
† LOD and LOQ, 5 ng/mL.
‡ LOD and LOQ, 1 ng/mL.
§ LOD, 10 ng/mL; LOQ, 25 ng/mL.

not affect identification or quantitation of the parent clobenzorex.

Evaluation of a quantitative procedure for 4-HO-clobenzorex allowed for accurate quantitation of the metabolite in samples. With the addition of a hydrolysis step, 4-HO-clobenzorex was easily detected using the same extraction, derivatization, and instrumental analysis parameters used for the determination of amphetamine. Figure 3 illustrates a typical chromatogram. An increase in clobenzorex concentrations in hydrolyzed samples compared to results from the same samples without hydrolysis was noted. However, under the GC conditions described here previously, the increase in clobenzorex concentrations may have been due to a co-eluting peak contributing to the peak area. Although an interesting observation, given the low clobenzorex levels observed, focusing on the 4-HO-clobenzorex to assess clobenzorex use is the most appropriate approach.

To determine extraction efficiency of 4-HO-clobenzorex, two sets of 12 samples were prepared with internal standard added to six samples prior to extraction and internal standard added to the remaining six samples after extraction. Results showed recoveries of the metabolite to be 22 and 24%. Although the extraction efficiency for 4-hydroxyclobenzorex appears at first glance to be low, the compound was readily extracted and found in high abundance during MS analysis. Consequently, detection and quantitation of the metabolite was easily accomplished even at low nanograms-per-milliliter concentrations. Linearity was defined as quantitative values within ± 20% of the target concentration with proper qualitative identification as determined by ion ratios within ± 20% and retention time within ± 2% of calibrator. The linear range was determined for this procedure by the analysis of at least seven different runs on concentrations ranging from 10 to 10,000 ng/mL of 4-HO-clobenzorex. Using the 500-ng/mL calibrator, samples ranging from 25 to 10,000 ng/mL provided results within ± 20% of their target value. Although the mean for the 10 ng/mL was within acceptable range, four of the individual sample results were not within the acceptable ± 20% limit; therefore, the LOQ was defined as 25 ng/mL. The limit of detection was shown to be 10 ng/mL (acceptable ion ratios, acceptable retention time, signal-to-noise ratio ≥ 3). Within-run precision was determined by testing replicates (n = 6) of the 4-HO-clobenzorex at 500 ng/mL and internal standard at 500 ng/mL in a single assay. Between-run precision was determined by calculating the mean, standard

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Table III. (continued) Analytical Results Following Single-Dose Administration of Clobenzorex*

Subject	Hours post dose	pH	Specific gravity	Concentration (ng/mL) Creatinine	Amp†	Clob‡	4-HO-clob§
2	27:00	5.76	1.022	336	450	0.0	109
2	28:45	5.69	1.021	193	196	0.0	53
2	31:00	5.08	1.023	326	441	0.0	92
2	32:50	5.18	1.019	150	279	0.0	68
2	35:00	5.95	1.020	192	166	0.0	Detected
2	37:55	6.88	1.020	282	94	0.0	Detected
2	45:30	5.26	1.026	368	245	0.0	Detected
2	48:40	6.35	1.023	184	81	0.0	Detected
2	51:05	5.73	1.021	181	20	0.0	0
2	53:30	5.44	1.015	100	17	0.0	0
2	56:10	5.61	1.020	189	55	0.0	0
2	58:50	5.71	1.026	366	63	0.0	0
2	60:40	6.60	1.025	201	14	0.0	0
2	67:00	5.46	1.026	306	46	0.0	Detected
2	71:00	5.35	1.024	185	23	0.0	0
2	74:00	6.17	1.021	152	10	0.0	0
2	76:20	6.08	1.017	141	0	0.0	0
2	78:30	4.90	1.022	188	17	0.0	0
2	80:15	5.05	1.026	242	18	0.0	0
2	85:00	6.01	1.025	226	6	0.0	0
2	87:30	5.87	1.011	77	0	0.0	0
2	87:55	6.05	1.002	24	0	0.0	0
2	92:30	5.37	1.009	83	0	0.0	0
2	95:00	5.03	1.016	176	7	0.0	0
3	00:00	6.89	1.016	133	0	0.0	0
3	05:00	6.35	1.018	160	2233	21.5	16,671
3	06:05	6.16	1.005	32	8	0.0	308
3	11:30	6.83	1.021	205	949	0.0	1686
3	19:58	6.13	1.020	148	515	0.0	520
3	24:28	6.83	1.016	96	278	0.0	297
3	32:00	5.70	1.018	163	244	0.0	370
3	35:59	5.38	1.023	320	626	0.0	468
3	47:30	5.96	1.026	199	316	0.0	164
3	54:35	6.02	1.022	156	115	0.0	68
3	56:35	5.63	1.010	70	47	0.0	32
3	58:45	5.68	1.019	164	82	0.0	32
3	67:30	5.82	1.024	184	48	0.0	Detected
3	77:30	5.84	1.021	177	23	0.0	Detected
4	00:00	5.32	1.012	78	0	0.0	0
4	01:30	5.94	1.007	47	162	4.2	5705
4	02:30	5.63	1.006	40	488	2.6	1986
4	07:30	5.29	1.018	222	2399	10.1	2891
4	11:30	5.77	1.026	272	1521	2.8	2677
4	14:00	5.50	1.026	217	981	0.0	760
4	20:30	5.49	1.028	201	1149	0.0	489
4	23:00	5.97	1.023	131	435	0.0	296
4	25:30	5.97	1.020	111	432	0.0	121
4	28:00	5.57	1.026	208	498	0.0	114
4	30:30	6.42	1.029	209	238	0.0	66
4	33:00	5.68	1.025	148	116	0.0	92

* Data from samples following the last analyte-containing sample from each subject are not included in the table. None of these samples contained detectable amphetamine (Amp), clobenzorex (Clob), or 4-HO-clobenzorex (4-HO-clob).
† LOD and LOQ, 5 ng/mL.
‡ LOD and LOQ, 1 ng/mL.
§ LOD, 10 ng/mL; LOQ, 25 ng/mL.

deviation, and RSD at 500 ng/mL of the hydroxy metabolite and internal standard on seven consecutive runs. The RSDs were 1.3 and 5.5% respectively.

A summary of amphetamine, clobenzorex, and 4-hydroxyclobenzorex results from the study samples is given in Table I. The first detectable level of amphetamine (LOD = 5 ng/mL) ranged from 1:00 to 5:00 h postdose. The first amphetamine positive (≥ 500 ng/mL) was seen at 2:40 h, and the last positive at 37:00 h postdose. Peak concentrations of amphetamine were seen in samples collected 4:30 to 19:30 h postdose where the concentrations reached 715 to 2474 ng/mL. Amphetamine was last detected from 77:30 up to 116:00 h postdose. The first detectable level of clobenzorex (LOD = 1 ng/mL) ranged from 1:30 to 5:00 h postdose for all five subjects with peak concentrations ranging from 10 to 29 ng/mL and seen 2:40 to 7:30 h postdose. It was last detected from 5:00 to 29:00 h postdose. 4-HO-clobenzorex was detected at much higher concentrations and lasted for a longer period of time than the parent drug. 4-HO-Clobenzorex was first detected (LOD = 10 ng/mL) from 1:00 to 5:00 h postdose. Peak concentrations were seen in samples collected 1:30 to 5:00 h postdose where concentrations reached 5705 to 88,410 ng/mL. It was last detected from 45:30 to 91:30 h postdose (see Figure 4 for summary of detection times for each analyte); this is consistent with the finding of Maurer et al. (2) that detected a hydroxy metabolite for 56 to 84 h postdose using full scan MS analysis.

The percentage of clobenzorex metabolized to amphetamine has been reported to range from 5.9 to 15.4% of the initial dose (1). Analysis of these samples showed the 4-hydroxy metabolite was excreted in much higher amounts ranging from 10.1 to 40.4% of the initial dose of clobenzorex (Table II). Although substantially higher in amount, the 4-HO-clobenzorex was also excreted more rapidly than amphetamine, reaching much higher concentrations than amphetamine in the early hours following administration of the drug but dropping off more rapidly (Table III). One important finding was that all subject urine samples that contained amphetamine at concentrations of greater than or equal to 500 ng/mL also had detectable amounts of 4-HO-clobenzorex.

Another significant finding in this study was that the metabolite was found at substantially higher concentrations than amphetamine. Peak levels of amphetamine ranged from 715 to 2474 ng/mL, and peak levels of 4-HO-clobenzorex were 5705 to 88,410 ng/mL.

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Table III. (continued) Analytical Results Following Single-Dose Administration of Clobenzorex*

Subject	Hours post dose	pH	Specific gravity	Concentration (ng/mL) Creatinine	Amp†	Clob‡	4-HO-clob§
4	34:30	5.87	1.020	89	142	0.0	41
4	36:30	5.65	1.019	89	173	0.0	Detected
4	38:30	5.42	1.023	136	242	0.0	30
4	45:30	5.25	1.024	154	196	0.0	Detected
4	48:30	5.23	1.026	170	137	0.0	0
4	49:30	5.44	1.026	199	108	0.0	0
4	53:30	5.55	1.024	166	60	0.0	0
4	56:30	5.88	1.028	212	46	0.0	0
4	58:00	6.15	1.011	47	13	0.0	0
4	60:00	5.98	1.021	145	28	0.0	0
4	66:00	5.46	1.020	109	28	0.0	0
4	68:00	5.15	1.018	91	20	0.0	0
4	71:00	5.38	1.012	85	12	0.0	0
4	72:00	6.31	1.003	16	0	0.0	0
4	72:30	5.99	1.004	24	0	0.0	0
4	74:00	5.38	1.012	81	11	0.0	0
4	75:30	5.55	1.007	50	0	0.0	0
4	76:30	6.07	1.005	40	0	0.0	0
4	78:00	5.95	1.004	38	0	0.0	0
4	80:30	5.71	1.009	89	6	0.0	0
4	83:00	5.64	1.018	180	9	0.0	0
4	91:30	5.60	1.023	201	9	0.0	0
5	00:00	5.53	1.022	159	0	0.0	0
5	01:00	7.02	1.004	15	23	0.0	513
5	03:00	6.63	1.010	61	1027	18.1	27,249
5	06:00	5.43	1.012	82	2232	2.5	3284
5	10:30	7.54	1.013	81	467	0.0	1207
5	13:00	6.38	1.009	54	968	0.0	839
5	19:30	6.43	1.016	100	1104	0.0	612
5	22:30	5.77	1.009	50	689	0.0	217
5	29:00	5.14	1.024	236	2038	1.2	799
5	32:00	5.61	1.025	180	1154	0.0	508
5	37:00	6.21	1.023	137	699	0.0	238
5	43:30	6.75	1.027	148	305	0.0	116
5	47:30	6.43	1.021	96	207	0.0	56
5	49:30	6.88	1.008	30	91	0.0	Detected
5	52:00	7.58	1.007	32	37	0.0	40
5	55:00	6.57	1.006	31	41	0.0	Detected
5	56:00	6.96	1.007	35	46	0.0	Detected
5	60:00	5.55	1.010	61	87	0.0	Detected
5	67:30	5.86	1.014	89	72	0.0	Detected
5	71:00	5.01	1.013	60	62	0.0	Detected
5	73:30	7.41	1.011	47	18	0.0	Detected
5	76:00	5.48	1.006	36	27	0.0	0
5	79:30	5.69	1.017	100	48	0.0	Detected
5	81:30	6.58	1.005	24	10	0.0	0
5	85:30	6.70	1.013	73	29	0.0	0
5	91:30	6.37	1.022	120	28	0.0	Detected
5	96:30	6.39	1.016	84	15	0.0	0
5	100:00	6.10	1.013	71	14	0.0	0
5	104:00	7.07	1.012	61	9	0.0	0

* Data from samples following the last analyte-containing sample from each subject are not included in the table. None of these samples contained detectable amphetamine (Amp), clobenzorex (Clob), or 4-HO-clobenzorex (4-HO-clob).
† LOD and LOQ, 5 ng/mL.
‡ LOD and LOQ, 1 ng/mL.
§ LOD, 10 ng/mL; LOQ, 25 ng/mL.

Although the 4-HO-clobenzorex peak concentrations far exceeded those for amphetamine, it also was excreted more rapidly than was amphetamine. In all subjects, 4-HO-clobenzorex could not be detected in any sample after amphetamine had dropped below detection limits. In all but one subject, amphetamine could be detected after the 4-HO-clobenzorex was no longer detectable. The 4-HO-clobenzorex was seen at higher concentrations for the first 10–32 h postdose, with peak concentrations ranging from 2.3 to 51 times that of amphetamine. After this point, a crossover occurred where amphetamine was most often found in greater amounts than 4-HO-clobenzorex, although at low concentrations (< 100 ng/mL), the concentration of 4-HO-clobenzorex sometimes again exceeded that of amphetamine. In every case however, high concentrations of 4-HO-clobenzorex were indicative of recent use. Evaluation of amphetamine concentration relative to the concentration of 4-HO-clobenzorex can be very valuable in estimation of time since dose (Table III). The variability of peak concentrations among subjects seemed to correspond with the variation in creatinine, pH, and specific gravity rather than being reflective of significant differences in metabolism. This is supported by analysis of changes in amphetamine and 4-HO-clobenzorex concentrations within individual subject samples. For each subject, dramatic changes in the urine concentration of amphetamine and 4-HO-clobenzorex corresponded with a change in the pH or creatinine and specific gravity consistent with the expected effect on excretion these parameters represent.

Conclusions

As previously reported in the single-dose study (1), administration of clobenzorex resulted in urinary excretion of amphetamine and variable but small amounts of the parent drug. Peak concentrations of amphetamine reached as high as 2474 ng/mL with detectable levels (≥ 5 ng/mL) seen for up to 116 h postdose. Although amphetamine was detected at relatively high concentrations, clobenzorex was detected at very low concentrations, for a relatively short period of time and was not detected in all amphetamine-positive samples (≥ 500 ng/mL). Thus, the absence of clobenzorex in the urine does not exclude the possibility of its use. In search for a better indicator of clobenzorex use, a metabolite was considered.

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Previously, qualitative identification of two hydroxy metabolites of clobenzorex was described. In at least one subject, one of the hydroxy metabolites was detected as long as amphetamine was present in the urine (2). Although in the present study, amphetamine could be detected in some samples after the 4-HO-clobenzorex metabolite could no longer be detected, the results seem consistent because of the differences in detection limits for the analytes in the two studies. In addition, in those cases in which amphetamine could be detected after the 4-HO-clobenzorex metabolite could no longer be detected, the concentration of amphetamine was far below the positive cutoff.

Acid hydrolysis in an autoclave for 30 min was shown to provide the highest release of the metabolite. Periodate was shown to have a significant negative effect on detection of the hydroxy metabolites. A procedure for the quantitation of 4-HO-clobenzorex was evaluated and proved to be viable for the detection of this metabolite in subject urine samples. Samples obtained from a controlled single-dose study were analyzed for the presence of the 4-hydroxy metabolite. 4-HO-Clobenzorex was found at much higher concentrations and could be detected for a much longer period of time than the parent drug. Interpretation of analytical results can be extremely helpful in determining the possibility of involvement of clobenzorex use. One would expect to find the enantiomeric composition of amphetamine to be that of the *d*-enantiomer only (1). Another important finding is that all samples containing amphetamine at or above the cutoff of 500 ng/mL showed detectable amounts of 4-HO-clobenzorex. One would also expect to see 4-HO-clobenzorex at higher concentrations than amphetamine for the first hours postadministration. Sometime after the first 10–32 h, amphetamine concentrations generally exceed the concentration of this metabolite. The metabolite, 4-HO-clobenzorex proved to be an excellent indicator of clobenzorex use compared to detection of the parent drug. Data presented here can help differentiate between use of clobenzorex and abuse of amphetamine.

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A Gas Chromatography–Mass Spectrometry Method for the Quantitation of Clobenzorex*

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Abstract

Drugs metabolized to amphetamine or methamphetamine are potentially significant concerns in the interpretation of amphetamine-positive urine drug-testing results. One of these compounds, clobenzorex, is an anorectic drug that is available in many countries. Clobenzorex (2-chlorobenzylamphetamine) is metabolized to amphetamine by the body and excreted in the urine. Following administration, the parent compound was detectable for a shorter time than the metabolite amphetamine, which could be detected for days. Because of the potential complication posed to the interpretation of amphetamine-positive drug tests following administration of this drug, the viability of a current amphetamine procedure using liquid–liquid extraction and conversion to the heptafluorobutyryl derivative followed by gas chromatography–mass spectrometry (GC–MS) analysis was evaluated for identification and quantitation of clobenzorex. Qualitative identification of the drug was relatively straightforward. Quantitative analysis proved to be a far more challenging process. Several compounds were evaluated for use as the internal standard in this method, including methamphetamine-d_{11}, fenfluramine, benzphetamine, and diphenylamine. Results using these compounds proved to be less than satisfactory because of poor reproducibility of the quantitative values. Because of its similar chromatographic properties to the parent drug, the compound 3-chlorobenzylamphetamine (3-Cl-clobenzorex) was evaluated in this study as the internal standard for the quantitation of clobenzorex. Precision studies showed 3-Cl-clobenzorex to produce accurate and reliable quantitative results (within-run relative standard deviations [RSDs] < 6.1%, between-run RSDs < 6.0%). The limits of detection and quantitation for this assay were determined to be 1 ng/mL for clobenzorex.

Introduction

Clobenzorex (2-chlorobenzylamphetamine) is an anorectic drug that is not available in the United States but is available in many countries as a prescription (e.g., Mexico) or over-the-counter (e.g., Panama) drug. Clobenzorex is one of several compounds metabolized to amphetamine by the body and excreted in the urine (1–4). Following administration, the parent compound was detectable, but the metabolite amphetamine was detectable for a longer period of time (2,5). Because of complications with interpretation of amphetamine-positive drug tests, the viability of a current amphetamine procedure (6) was evaluated for identification and quantitation of this compound. The method employs a liquid–liquid extraction followed by derivatization with heptafluorobutyric anhydride (HFBA) and GC–MS analysis. Several published studies describe procedures for the identification of clobenzorex (2,5,7–10). Few of these methods describe quantitation of clobenzorex. Those that do, unfortunately, do not describe the quantitative accuracy or precision of the method but rather describe the detection of the drug and/or its metabolite(s). Maurer et al. (2) reported a procedure for the detection of clobenzorex and a number of its metabolites, including amphetamine, and the time course of their detection. This method had a limit of detection in urine of 50 ng/mL for clobenzorex and 100 ng/mL for amphetamine. However, after administration of 60 mg clobenzorex, subject urine samples were shown to contain no detectable clobenzorex after the first 4 h, but amphetamine was detected for 60 h.

Materials

Amphetamine, methamphetamine, and methamphetamine-d_{11} (1-phenyl-d_5-2-methyl-d_3-aminopropane-3,3,3-d_3) were obtained from Radian Corp. Amphetamine-d_6 (1-phenyl-2-aminopropane-1,1,2,3,3,3-d_6) and trifluoroacetic anhydride (TFAA) were obtained from Alltech. Heptafluorobutyryl chloride (HFB.Cl) was obtained from Aldrich Chemical Co. Clobenzorex hydrochloride was obtained through colleagues in Spain. The internal standard, 3-chlorobenzylamphetamine (3-Cl-clobenzorex), was synthesized in our laboratory. Heptafluorobutyric anhydride (HFBA), fenfluramine hydrochloride, benzphetamine hydrochloride, and diphenylamine were obtained from Sigma Chemical Co. Urine samples were from a metabolic study evaluating conversion of clobenzorex to amphetamine following the administration of 30 mg clobenzorex (Asenlix, Roussel) to experimental subjects (11).

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Synthesis of 3-Cl clobenzorex was accomplished using the procedure described by Lintermans et al. (12).

Methods

Sample preparation

Control samples at various concentrations were prepared by dilutions with drug-free urine from a 100 µg/mL clobenzorex in methanol stock solution to concentrations that ranged from 1 to 500 ng/mL. Samples (2 mL) were aliquoted into glass tubes and refrigerated prior to analysis.

Sample analysis

The procedure used for the analysis of these samples is based on a previously described method for the analysis of amphetamine and methamphetamine (6). This method is linear for amphetamine from 5 to 10,000 ng/mL with a limit of detection (LOD) of 5 ng/mL. Minor modifications were made to acquire the appropriate ions for monitoring clobenzorex and internal standards. In addition, the temperature program was increased following the elution of methamphetamine to decrease the retention time of clobenzorex and shorten the total run time. Details of the method are described.

Extraction procedure. Two-milliliter urine samples were aliquoted into glass tubes followed by the addition of 0.3 mL 1M NaOH and 5 mL 1-chlorobutane. Addition of 1.0 mL of 0.35M sodium periodate allowed to stand at room temperature for 15 min to eliminate potential interference from related hydroxy compounds is optional at this point in the procedure. Tubes were placed on a shaker for 10 min at 120 cycles/min (cpm) then centrifuged for 5 min at 1500 rpm in a Sorvall RC3C centrifuge to separate the layers. The organic layer was transferred to a clean, dry glass tube followed by the addition of 2.0 mL 0.15M sulfuric acid. Tubes were shaken and centrifuged as described. The top organic layer was aspirated to waste followed by the addition of 1 mL of 1M NaOH and 5 mL 1-chlorobutane to the bottom aqueous layer. Samples were then shaken and centrifuged as described. The top organic layer was then transferred to a clean, dry glass tube to which 200 µL of 1.0% HCl in methanol was added. Samples were then placed in a water bath (50–60°C) and evaporated to dryness under a stream of nitrogen. The dried extract was reconstituted in 100 µL of ethyl acetate, 25 µL of HFBA was added, and the mixture incubated at 60–70°C for 15 min, then evaporated to dryness under nitrogen. The dried extract was reconstituted in ethyl acetate and 1–2 µL was injected on the GC–MS for analysis.

Instrumental analysis. GC–MS analysis were performed using a Hewlett-Packard 5890 II GC coupled with an HP 5971 MS. GC conditions were as follows: splitless injection using a 7673 autoinjector with injection port and interface temperatures set at 270°C. Conditions for the HP-1 column (12 m × 0.2-mm i.d., 0.33-µm film thickness, Hewlett-Packard) consisted of a temperature program with an initial time of 1 min at 80°C, programmed to 180°C at 20°C/min, then programmed to 250°C at 28°C/min with a 1 min final time. Conditions for the DB-17 column (30 m × 0.18-mm i.d., 0.30-µm film thickness, J&W Scientific) were the same as for the HP-1 with the exception of a final time of 2.0 min.

Quantitative analysis. Quantitation and detection of amphetamine and methamphetamine was based on single-point calibration using a calibration standard containing 500 ng/mL clobenzorex and 250 ng/mL of their internal standards as previously described (11). The calibration standard also contained 25 ng/mL of clobenzorex and 50 ng/mL of 3-chloroclobenzorex as internal standard. Low-concentration samples were quantitated based on single-point calibration using a calibration standard at 25 ng/mL of amphetamine and methamphetamine together with 10 ng/mL of clobenzorex and 50 ng/mL of all internal standards. Ions monitored were amphetamine: *m/z* 240, 118, 91; methamphetamine: *m/z* 254, 210, 118; amphetamine-d_6: *m/z* 244, 123; and methamphetamine-d_{11}: *m/z* 260, 213 with the additional monitoring of ions for clobenzorex and 3-Cl-clobenzorex at *m/z* 91, 118, 125, and 364, following the elution of methamphetamine. Ions monitored for other internal standards include fenfluramine: *m/z* 159, 240, 268; benzphetamine: *m/z* 65, 91, 148; and diphenylamine: *m/z* 77, 117.

Acceptance criteria. The criteria used in the evaluation of analytical results included quantitative values within ± 20% of the target concentration with proper qualitative identification as determined by ion ratios within ± 20% and retention time within ± 2% of the calibration standard while exhibiting acceptable chromatography.

Results and Discussion

Several attempts were made to quantitate clobenzorex using methamphetamine-d_{11}, fenfluramine, or benzphetamine as internal standards. The results using these compounds proved to be unacceptable because of poor reproducibility of the quantitative values. Along with investigating these compounds as potential internal standards, additional procedure modifications were evaluated. The use of HFBA and other derivatizing reagents (TFAA, HFB.Cl) at higher temperatures (70–80°C) in the derivatization step did not improve quantitative results. A solid-phase extraction procedure using C18 cartridges and methamphetamine-d_{11} or fenfluramine (10) as internal standard was evaluated, but this procedure failed to produce accurate quantitative results as well. Diphenylamine was also considered for the internal standard; however, poor reproducibility was immediately noted after just two runs, so no further evaluation of this compound was completed. Because of the poor quality of the results from these compounds, other options were considered. In the evaluation of possible candidates for a suitable internal standard, consideration was given to compounds that would have the closest physiochemical properties to clobenzorex. Short of a deuterium-labeled isotopomer, regioisomers of clobenzorex would logically have the closest fit. Chromatographic behavior of the regioisomers of clobenzorex has been described by Noggle et al. (13). HPLC using a nonpolar C18 column showed the greatest separation between the 2-chloro and 3-chloro isomers with the 4-chloro isomer eluting between the two. For this reason, the compound 3-Cl-clobenzorex was synthesized and evaluated in this study as an internal standard for the quantitation of clobenzorex.

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Figure 1 shows the structure of 3-Cl-clobenzorex, revealing its relationship to clobenzorex. The compound proved to have similar extraction and derivatization behaviors to clobenzorex and was chromatographically separated from the drug on a nonpolar (HP-1) and a more polar (DB-17) GC capillary column. Clobenzorex was shown to elute after methamphetamine (relative retention time, 1.58 on HP-1, 1.66 on DB-17). Typical chromatographic performance on an HP-1 column for amphetamine, methamphetamine, clobenzorex, and 3-Cl-clobenzorex is shown in Figure 2.

Linearity was defined as quantitative values within ± 20% of the target concentration with proper qualitative identification as determined by ion ratios within ± 20% and retention time within ± 2%. The linear range was determined for this procedure by the analysis of at least seven different runs on concentrations ranging from 1 to 500 ng/mL clobenzorex. The results of this evaluation are shown in Table I. Evaluation of higher concentrations was not undertaken because the subject samples tested contained clobenzorex only at low levels. Using the 10-ng/mL calibrator, samples ranging from 1 to 50 ng/mL provided average results within ± 20% of their target value. The use of a calibrator at 25 ng/mL yielded a linear range from 5 to 250 ng/mL based on the average of measured results. Examination of the data in Table I shows that at the higher levels, there is a negative bias that could presumably be obviated by calibration at a higher level or by use of a multi-point calibration curve. Using a single-point calibrator, however, the most accurate results were from 1 to 25 and 5 to 100 ng/mL for the 10- and 25-ng/mL calibrators, respectively. The use of a quantitative method with the ability to accurately quantitate concentrations as low as 1 ng/mL was of value in this study in order to allow for the accurate evaluation of the amount of parent drug excreted. Under normal circumstances, the ability to merely

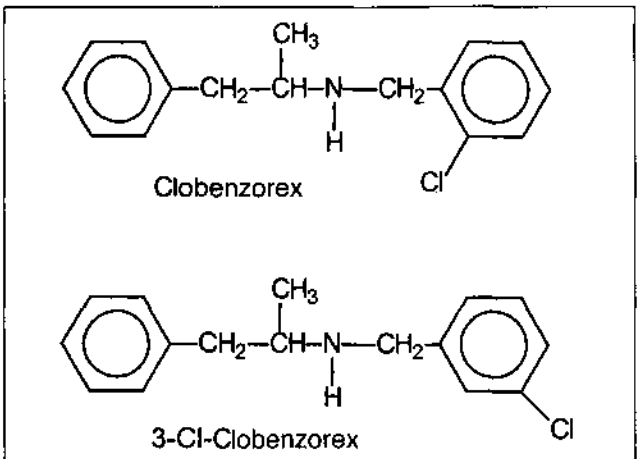

Figure 1. Chemical structures of clobenzorex and 3-Cl-clobenzorex.

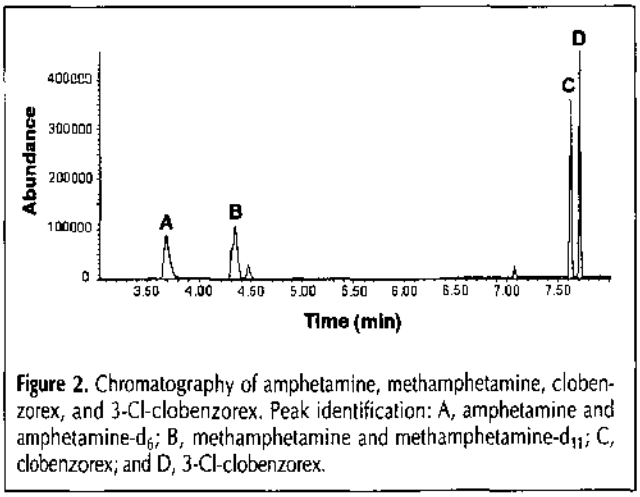

Figure 2. Chromatography of amphetamine, methamphetamine, clobenzorex, and 3-Cl-clobenzorex. Peak identification: A, amphetamine and amphetamine-d_6; B, methamphetamine and methamphetamine-d_{11}; C, clobenzorex; and D, 3-Cl-clobenzorex.

Table I. Linearity Data from Analysis of Samples at Specified Concentrations

Concentration	Average	SD	RSD	Difference
10 ng/mL Calibration				
0	0.00	–	–	–
1	1.11	0.06	5.0%	11.0%
2	2.10	0.19	9.0%	5.0%
3	3.10	0.19	6.1%	3.3%
4	4.13	0.15	3.7%	3.3%
5	4.99	0.22	4.4%	–0.2%
25	21.92	0.70	3.2%	–12.3%
50	40.28	1.69	4.2%	–19.4%
100	76.70	2.65	3.5%	–23.3%
200	137.20	7.24	5.3%	–31.4%
250	171.45	7.68	4.5%	–31.4%
500	298.72	28.69	9.6%	–40.3%
25 ng/mL Calibration				
0	0.00	–	–	–
1	1.29	0.07	5.2%	29.0%
2	2.47	0.23	9.0%	23.5%
3	3.62	0.29	7.9%	20.7%
4	4.90	0.23	4.8%	22.5%
5	5.84	0.51	8.6%	16.8%
10	11.79	0.51	4.3%	17.9%
25	25.50	2.51	9.6%	2.0%
50	47.60	3.35	7.0%	–4.8%
100	91.23	5.21	5.7%	–8.8%
200	161.03	15.89	9.7%	–19.5%
250	202.78	13.33	6.5%	–18.9%
500	369.63	23.61	6.3%	–26.1%

Table II. Precision

Internal standard (50 ng/mL)	*n*	100 ng/mL Clobenzorex* (RSD)	5 ng/mL Clobenzorex† (RSD)
Between-run			
3-Cl-clobenzorex	8	5.7%	4.4%
methamphetamine-d_{11}	8	51.1%	
fenfluramine	6	33.1%	
benzphetamine	8	28.5%	
diphenylamine	2	39.1%	
Within-run			
3-Cl-clobenzorex	6	3.0%	6.1%

* Precision determined using a 25-ng/mL calibrator.
† Data determined using a 10-ng/mL calibrator.

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detect the drug at such low levels allows for clear evaluation of the use of clobenzorex and accurate quantitation is not a requirement. At the same time, the levels detected were quite low from the single administration of this drug and having a higher linear range was not necessary in these cases. In multiuse or abuse levels, higher levels may well be encountered, making the calibration at 25 ng/mL a more useful choice, particularly because the absolute concentration of the drug between 1 and 5 ng/mL provides little additional useful information to the fact that the drug is present.

Precision was determined by testing replicates of clobenzorex at 5 and 100 ng/mL and internal standard (3-Cl-clobenzorex) at 50 ng/mL. Within-run precision was determined by testing replicates (n = 6) of the drug at 100 ng/mL using the 25-ng/mL calibrator and at 5 ng/mL drug using the 10-ng/mL calibrator in a single assay. The relative standard deviations (RSD) were 3.0 and 6.0%, respectively. Between-run precision was determined by calculating the mean, standard deviation, and RSD at 100 ng/mL of drug (using 25 ng/mL calibrator), 5 ng/mL of drug (using 10 ng/mL calibrator), and 50 ng/mL internal standard on eight consecutive runs. The RSDs were 5.7 and 4.4%, respectively. Between-run precision using 50 ng/mL of each internal standard, methamphetamine-d_{11}, fenfluramine, benzphetamine, and diphenylamine, was also determined. The RSDs for these compounds were all > 25% and deemed inferior to 3-Cl-clobenzorex for use as internal standards. The between-run precision for 100 ng/mL clobenzorex and 50 ng/mL of all internal standards tested in this assay can be seen in Table II.

Table III. Clobenzorex Concentrations

Subject	Hours postdose	Concentration (ng/mL) Amphetamine	Clobenzorex	Subject	Hours postdose	Concentration (ng/mL) Amphetamine	Clobenzorex
1	Predose	0	0	3	05:00	**2233**	21
1	01:30	0	0	3	06:05	8	0
1	04:30	**715**	12	3	11:30	**949**	0
1	07:30	**535**	4	3	19:58	**515**	0
1	08:30	100	0	3	24:28	278	0
1	10:00	60	0	3	32:00	244	0
1	12:30	129	0	3	35:59	**626**	0
1	14:00	46	0	3	47:30	316	0
1	17:00	116	0				
1	20:00	315	0	4	Predose	0	0
1	23:00	264	0	4	01:30	162	4
1	25:00	82	0	4	02:30	488	3
1	26:40	47	0	4	07:30	**2399**	10
1	29:30	59	0	4	11:30	**1521**	3
1	33:00	136	0	4	14:00	**981**	0
1	35:00	29	0	4	20:30	**1149**	0
1	37:00	80	0	4	23:00	435	0
1	41:47	49	0	4	25:30	432	0
1	47:00	55	0	4	28:00	498	0
				4	30:30	238	0
2	Predose	0	0	4	33:00	116	0
2	02:40	**1331**	29	4	34:30	142	0
2	04:45	398	2	4	36:30	173	0
2	06:35	**1721**	14	4	38:30	242	0
2	07:25	469	0	4	45:30	196	0
2	08:40	469	0				
2	10:00	389	0	5	Predose	0	0
2	12:20	420	0	5	01:00	23	0
2	19:30	**2474**	2	5	03:00	**1027**	18
2	23:00	**1187**	0	5	06:00	**2232**	2
2	27:00	450	0	5	10:30	467	0
2	28:45	196	0	5	13:00	**968**	0
2	31:00	**441**	0	5	19:30	**1104**	0
2	32:50	279	0	5	22:30	**689**	0
2	35:00	166	0	5	29:00	**2038**	1
2	37:55	94	0	5	32:00	**1154**	0
2	45:30	245	0	5	37:00	**699**	0
				5	43:30	305	0
3	Predose	0	0	5	47:30	207	0

* Results from samples taken from the first (approximately) 48 h postdose following administration of a single dose of clobenzorex. None of the samples collected after this time contained detectable clobenzorex using the method described in this paper. Bold numbers indicate those samples that would be positive using a 500-ng/mL cutoff.

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The limit of quantitation (LOQ) was based on data showing results within ± 20% of the target. The LOQ was determined by the analysis of a number of samples containing drug and internal standard. Clobenzorex samples prepared at concentrations of 1, 2, 3, and 4 ng/mL were used to assess the assays ability to detect and quantitate the drug at low concentrations. When using a 25 ng/mL calibrator and 50 ng/mL of internal standard, ion ratios passed and acceptable signal-to-noise ratios (S/N > 3:1) at 1 ng/mL were observed, however, quantitative results were unacceptable for levels below 5 ng/mL. Thus, the LOQ was determined to be 5 ng/mL and the limit of detection (LOD) 1 ng/mL. When a 10-ng/mL calibrator and 50-ng/mL internal standard were used, these low-concentration samples gave acceptable quantitative results, as well as acceptable ion ratios and signal-to-noise ratios (S/N > 10:1 at 1 ng/mL). Therefore, the LOD and LOQ for this assay, using a 10-ng/mL calibrator, is 1 ng/mL.

Twelve samples were analyzed to determine the efficiency of the extraction procedure. Clobenzorex (100 ng/mL) was added to all samples, and internal standard was added to six of the samples prior to extraction. Internal standard was added to the remaining six samples after extraction. The mean and standard deviation were calculated for each set of samples and extraction efficiency determined. Clobenzorex recovery was calculated relative to the internal standard 3-Cl-clobenzorex and determined to be 82 ± 8%. The recovery is similar to the 85% reported in another study (2). Six different random urine matrices were evaluated to check for any indication of interference with the monitored ions for the drug or internal standards under the conditions used in this study. Evaluation of these matrices using full scan spectra and extracted ions in the retention time range of clobenzorex gave no indication of interference with the drug. HP-1 and DB-17 capillary columns were evaluated to allow their use to eliminate potential interference from related amines in samples that contained substantial amounts of these compounds.

The full scan mass spectra of clobenzorex is shown in Figure 3. Clobenzorex was monitored by acquiring four prominent ions in the mass spectrum at *m/z* 91, 118, 125, and 364. All four ions were evaluated to assess the best three ions to select for routine monitoring. Interference was occasionally seen at *m/z* 91 and/or *m/z* 118, but in most cases, either ion gave acceptable results.

Application of this procedure to urine samples from a study which involved administration of clobenzorex to individuals revealed the amount of clobenzorex was quite low in all of the samples containing the drug. Table III shows the results obtained on analysis of the urine samples for the presence of amphetamine and clobenzorex. With a detection limit of 1 ng/mL, this study reveals clobenzorex to be detectable for longer periods (up to 29 h postdose) than previously reported. This is not a surprising finding given the substantially higher detection limits of the other studies. Although this does help significantly with the interpretation of amphetamine results, there were still samples that were positive (≥ 500 ng/mL) for amphetamine but contained no detectable clobenzorex. This fact complicates interpretation of the amphetamine-positive results. The concentrations of amphetamine and clobenzorex and the length of time they are detected does provide some useful information for purpose of interpretation. The fact that the amphetamine produced from clobenzorex is the *d*-enantiomer is also important information (11). Unfortunately, the fact remains that samples positive for amphetamine caused by the use of clobenzorex may not contain the parent drug.

This study did not include a hydrolysis step. Previously, qualitative identification of an as yet unidentified hydroxy metabolite of clobenzorex showed the presence of this metabolite, in at least one subject, to be detected as long as amphetamine was present in the urine (2). Without hydrolysis, it appeared that there was not a substantial amount of this hydroxy metabolite extracted from the urine. This is not a surprising result assuming that the metabolite is conjugated and nothing in the current procedure would cause the hydrolysis of the conjugate. Further study assessing the presence and concentrations of unique metabolites is needed to obtain additional information to appropriately address the possible involvement of this drug.

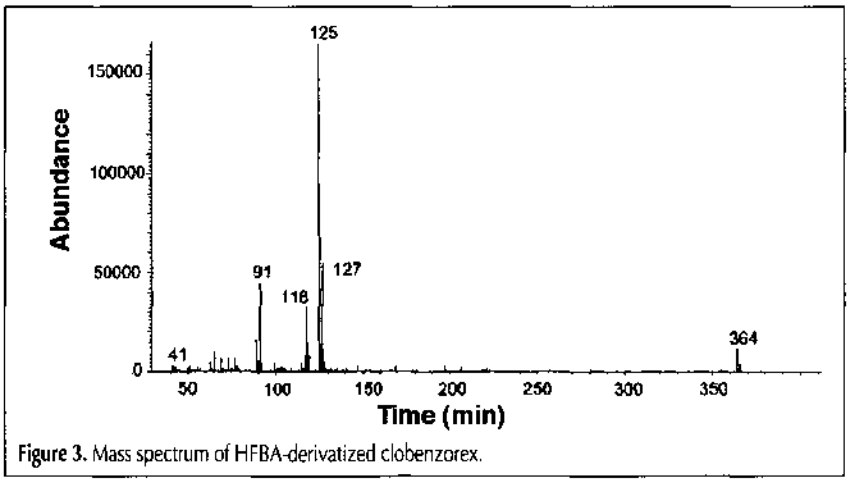

Figure 3. Mass spectrum of HFBA-derivatized clobenzorex.

Conclusions

Drugs such as clobenzorex that are metabolized to amphetamine are potentially significant concerns in the interpretation of amphetamine-positive drug-testing results. Interpretation of analytical results can be helpful in determining the possibility of involvement of this drug. In attempts to provide a quantitative method for clobenzorex, several internal standards were evaluated. However, poor reproducibility made most of these compounds a poor choice. 3-Cl-Clobenzorex proved to be the best choice as an internal standard because it provided the most consistent results for the quantitation of clobenzorex. Evaluation of assay characteristics using a minor modification of an existing amphetamine procedure proved to be viable and is presented here to assist laboratories with identification and quantitation of clobenzorex.

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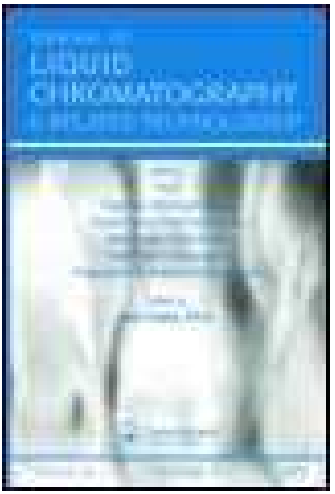

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Liquid Chromatographic Analysis of Regioisomers and Enantiomers of N-(Chlorobenzyl)-α-Methylphenethylamines: Analogues of Clobenzorex

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JOURNAL OF LIQUID CHROMATOGRAPHY, 13(4), 763-777 (1990)

LIQUID CHROMATOGRAPHIC ANALYSIS OF REGIOISOMERS AND ENANTIOMERS OF N-(CHLOROBENZYL)-α-METHYLPHENETHYLAMINES: ANALOGUES OF CLOBENZOREX

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ABSTRACT

The regioisomeric 2-, 3- and 4-chlorobenzylamphetamines were synthesized from racemic and (+)-amphetamine by reductive alkylation. The 2-, 3- and 4-chloro regioisomers were separated by reversed-phase liquid chromatography following phenylisothiocyanate derivatization. The indiviual enantiomers of each regioisomer were identified by HPLC following derivatization with GITC. Normal phase liquid chromatographic analysis of the diastereomeric GITC derivatives produced α-values of approximately 1.0 for each racemic pair of regioisomers. These methods were developed in order to specifically identify the drug clobenzorex, d-N-(2-chlorobenzyl)-α-methylphenethylamine and distinguish it from its optical and regioisomers.

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INTRODUCTION

Low molecular weight amines structurally related to amphetamine are present in a variety of formulations and street drug samples, and these compounds continue to be a challenging area of pharmaceutical and forensic analysis [1-5]. Designer drug modifications of many of the more established drugs of abuse have produced a variety of new compounds which differ only slightly in their structure in comparison to the parent compound [1-5]. Modifications such as homologation or regioisomerism produce compounds which often require the development of specific analytical methods and/or techniques for their specific identification [1-4]. Additionally, the analysis of street drug samples to determine the enantiomeric composition of the active drug ingredients can often distinguish a diverted pharmaceutical dosage form from a clandestinely manufactured substance [6,7].

The N-2-chlorobenzyl derivative of d-amphetamine, N-(2-chlorobenzyl)-α-methylphenethylamine or 2-chlorobenzylamphetamine, is marketed in Mexico and is known as clobenzorex. This drug is used in therapy for its anorexic properties which are similar to those of amphetamine. The d-enantiomers of amphetamine type compounds have higher stimulant and anorexic potencies than the enantiomeric l-isomer [8]. Clobenzorex has been encountered in forensic samples in recent years, requiring specific methods for its identification. These methods should be able to distinguish clobenzorex (2-chlorobenzyl) from its 3- and 4-chloro regioisomeric derivatives, as well as the enantiomeric composition of the sample.

In this study we have prepared the (+)-enantiomers and racemic mixtures of the three chloro regioisomers of

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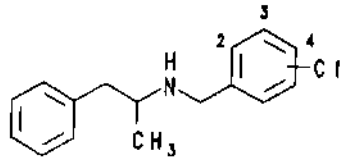
clobenzorex. The regioisomers were separated by reversed-phase liquid chromatography and the enantiomers were identified by normal phase chromatography following diastereomeric derivatization.



Clobenzorex (2-Cl) and its regioisomers

EXPERIMENTAL

Instrumentation. The liquid chromatograph consisted of a Waters Associates Model 6000A pump, U6K injector, Model 440 UV detector with dual wavelength accessory operated at 254, and a Houston Instruments OmniScribe dual pen recorder. Infrared spectra were recorded on a Perkin-Elmer Model 1710 Fourier transform infrared (FTIR) spectrophotometer. Ultraviolet spectra were recorded on a Shimadzu Instruments Model UV-160 spectrophotometer. Nuclear magnetic resonance spectra (1H) were obtained using a Varian EM360 spectrometer. The electron impact mass spectra were obtained using a Hewlett-Packard 5970B mass selective detector. The ionization voltage was 70 eV and the source temperature was 220°C. The individual samples were dissolved in methanol (1 mg/mL) and 0.5 uL introduced into the mass spectrometer via the gas chromatograph equipped with a 12m x 0.31 mm id fused silica column with a 0.52 um thickness of OV-1. The column temperature was programmed from 70°C to 150°C at a rate of 15°C/min and from

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150°C to 250°C at a rate of 25°C/min. The split ratio for the GC was 10:1 and all samples eluted in approximately 7 minutes.

Liquid Chromatographic Procedures. The reversed-phase analytical column was 30 cm x 3.9 mm id packed with uBondapak C_{18} (Waters Associates, Milford, MA). The analytical column was preceded by a 7cm x 2.1mm id guard column dry packed with CO:Pell ODS (Whatman). The amine-phenylisothiocyanate (PIT) derivatives were dissolved in HPLC grade methanol and chromatographed using a mobile phase of methanol, water and acetic acid (65:34:1). The mobile phase flow rate was 1.5 mL/min and the detector was operated at 1.0 AUFS.

The normal phase liquid chromatographic separations were accomplished using a 30 cm x 3.9 mm id u-Porasil (Waters Associates). The mobile phase was n-hexane, chloroform and 2-propanol (500:25:3) at 1.5 mL/min and the detector was operated at 254 nm and 0.2 AUFS.

Derivatization Procedures. PIT derivatives were prepared by dissolving 2 mg of the amine hydrochlorides in 30 mL of 0.45 N NaOH and extracting the free bases into 30 mL of chloroform. To this chloroform solution was added 10 uL of neat phenylisothiocyanate (PIT), and the reaction mixture evaporated to dryness under a stream of air. The resulting residue was dissolved in 1 mL of methanol and 5 uL of this solution was injected into the liquid chromatograph.

The GITC derivatives were prepared by first extracting the free bases into chloroform as described above, and then adding a 10% molar excess of GITC as a chloroform solution. After 10 minutes at room temperature this reaction mixture was evaporated

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to dryness under a stream of air. The resulting residue was dissolved in 1 mL of chloroform and 5 uL of this solution injected into the liquid chromatograph.

Synthesis of the Clobenzorex Derivatives. Racemic or (+)-amphetamine sulfate (685 mg, 1.86 mmol) was dissolved in water (25 mL) and the solution made basic (pH 12) with a saturated solution of NaOH. The basic aqueous solution was extracted with ether (3 X 25 mL) and the combined ether extracts dried over $MgSO_4$, filtered and the filtrate evaporated in vacuo to obtain the free base. The base was dissolved in absolute ethanol (20 mL), the appropriate chlorobenzaldehyde added (540 mg, 3.84 mmol) and the reaction mixture stirred at room temp for 18 - 20 hours. The ethanol solvent was then removed in vacuo to yield the intermediate imines as viscous yellow oils. The crude imines were dissolved in methanol (25 mL) and $NaBH_4$ (130 mg, 3.5 mmol) was added portionwise. The reaction mixture was then stirred at room temp for 2 hours, then at reflux for 1 hour. After cooling (room temp), the mixture was evaporated in vacuo and the residue suspended in water (25 mL). The aqueous suspension was acidified (pH 1) with dilute HCl, and then made alkaline (pH 12) with dilute NaOH. The basic suspension was then extracted with ether (3 X 25 mL) and the combined ether extracts dried ($MgSO_4$), filtered and the filtrate evaporated in vacuo to yield the product bases as pale yellow oils. Treatment of the bases with ethereal HCl afforded the hydrochloride salts which were isolated by filtration.

RESULTS AND DISCUSSION

The regioisomeric 2-, 3- and 4-chlorobenzylamphetamines were sythesized from racemic and (+)-amphetamine by reductive alkyla-

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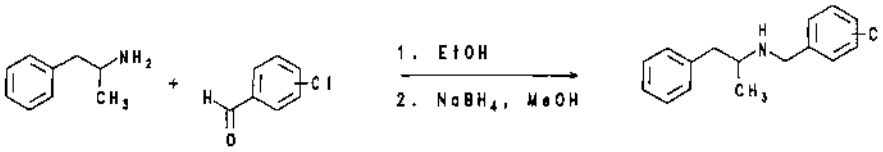

Scheme I. Synthesis of the regioisomeric N-(chlorobenzyl)-α-methylphenethylamines.

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tion as outlined in Scheme I [1,2]. Treatment of the free base of amphetamine with commercially available chlorobenzaldehydes yielded the intermediate imines. The crude imines were then reduced to the desired regioisomeric and enantiomeric chlorobenzylamphetamines with sodium borohydride in methanol. The bases of these compounds were converted to the corresponding hydrochloride salts with etheral hydrochloric acid. The structures of all products were confirmed by ultraviolet, infrared, proton NMR and mass spectral analysis.

The liquid chromatographic retention properties of clobenzorex and its regioisomers were quite similar under a variety of conditions. The underivatized amines were examined by reversed-phase techniques on both C_{18} and phenyl stationary phases with only limited success. The 2-chloro regiomer (clobenzorex) was well resolved in several systems, however the 3- and 4- isomers coeluted in all cases. Therefore, the amines were derivatized with phenylisothiocyanate (PIT) to form the phenylthiourea derivatives in an effort to improve the chromatographic properties of these compounds [9]. The isocratic reversed-phase separation of the PIT derivatives in Figure 1 was obtained with a C_{18} stationary phase and a mobile phase of methanol, water and acetic acid (65:34:1). The 3-chloro isomer has the

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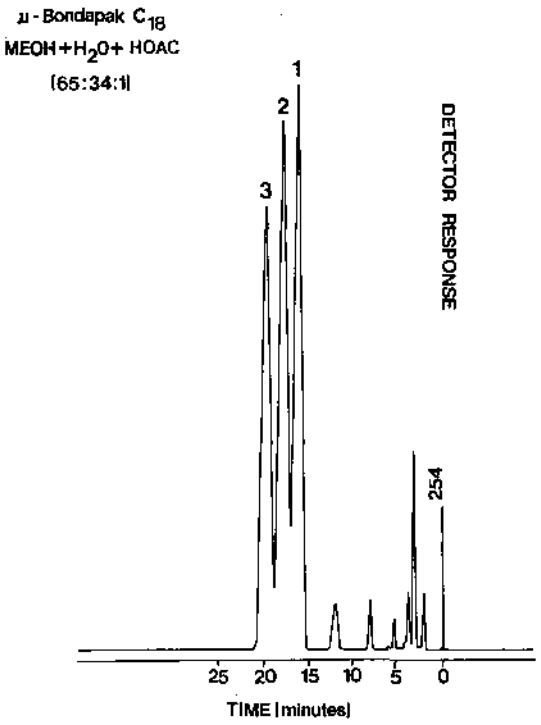

Figure 1. Reversed-phase liquid chromatographic separation of N-(chlorobenzyl)-α-methylphenethlyamines. Peaks: 1 = 3-chloro; 2 = 4-chloro and 3 = 2-chloro.

lowest capacity factor in this system followed by the 4-isomer, and clobenzorex (2-chloro isomer) has the highest capacity factor.

The underivatized amines were also examined by gas chromatography and the results were similar to those obtained by HPLC. The GC system was an OV-1 capillary column (12 m X 0.31 mm id) and a temperature gradient from 70°C to 250°C. The 3- and 4-chloro regiomers were unresolved in this and other variations of the temperature program.

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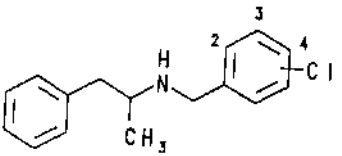
Table 1. Yields and melting points of the chlorobenzorex regioisomers.



Amine	Stereochem	Yield, % (base)	mp, °C (HCl)
2-Chloro	(+)	83	174-176[a]
3-Chloro	(+)	81	145-147
4-Chloro	(+)	79	203-204
2-Chloro	(±)	89	169-170
3-Chloro	(±)	93	164-164
4-Chloro	(±)	70	184-186

[a]The literature melting point for (+)-clobenzorex is 182 - 183°C.

Since the pharmaceutical dosage form of clobenzorex contains only the (+)-enantiomer, it is important to determine the enantiomeric composition of the sample. A variety of methods for the chiral separation of various drugs have been reported [10-14]. Most of these are liquid chromatographic methods making use of chiral stationary phases, chiral mobile phase additives or pre-column diastereomeric derivatization. In many applications, the chemical derivatization of the enantiomeric amines with chiral reagents to yield diastereomeric products offers the advantage of speed and convenience. Many chiral derivatizing agents are commercially available and the resulting diastereomeric products can

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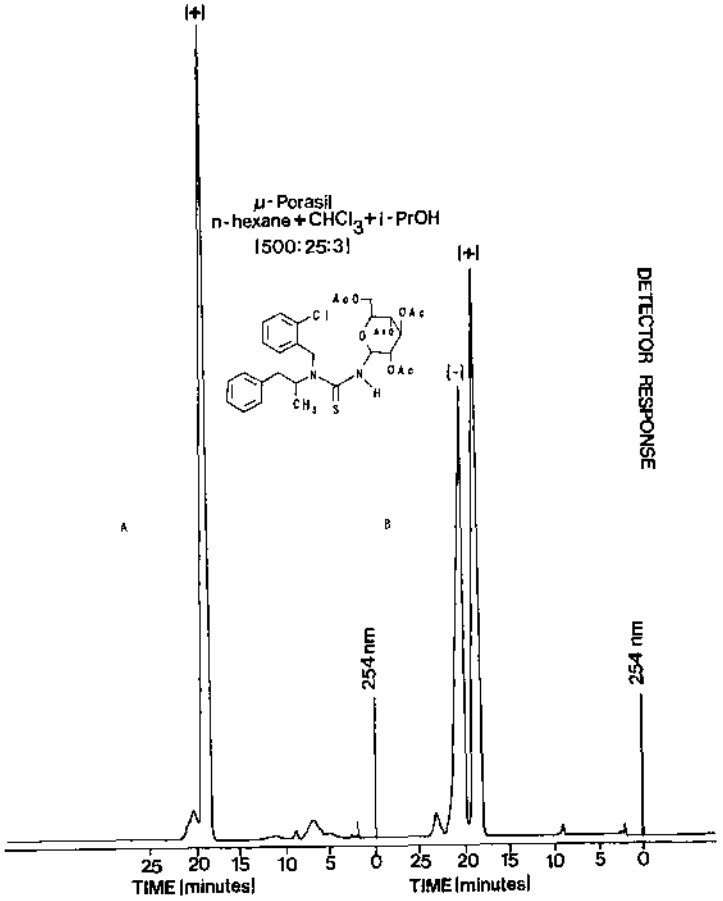

Figure 2. Normal-phase liquid chromatographic separation of GITC-derivatized enantiomers of N-(2-chlorobenzyl)-α-methylphenethylamine. A = (+)-enantiomer; B = racemic mixture.

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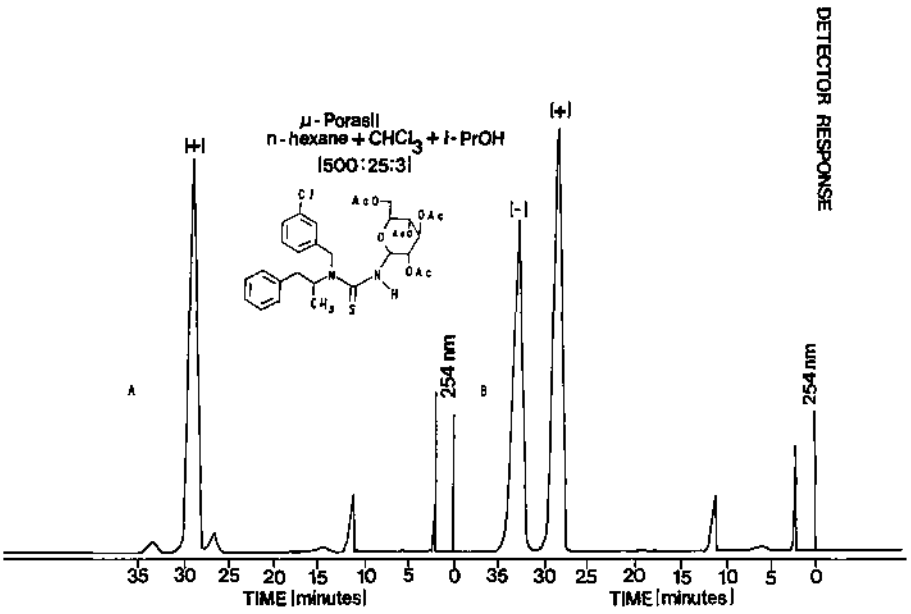

Figure 3. Normal-phase liquid chromatographic separation of GITC-derivatized enantiomers of N-(3-chlorobenzyl)-α-methylphenethylamine. A = (+)-enantiomer; B = racemic mixture.

be resolved on standard achiral stationary phases. Thus, the chiral analysis of a sample requires only the additional chiral derivatizing agent. One such agent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), has been used in forensic analysis for the derivatization of chiral drugs of abuse which are primary and secondary amines [6,7,15]. The derivatization of clobenzorex and its regioisomers was accomplished in chloroform solution at room temperature using a slight excess of GITC. The resulting thiourea diastereomers were separated by normal-phase chromatography on a silica column (μ-Porasil) and a mobile phase

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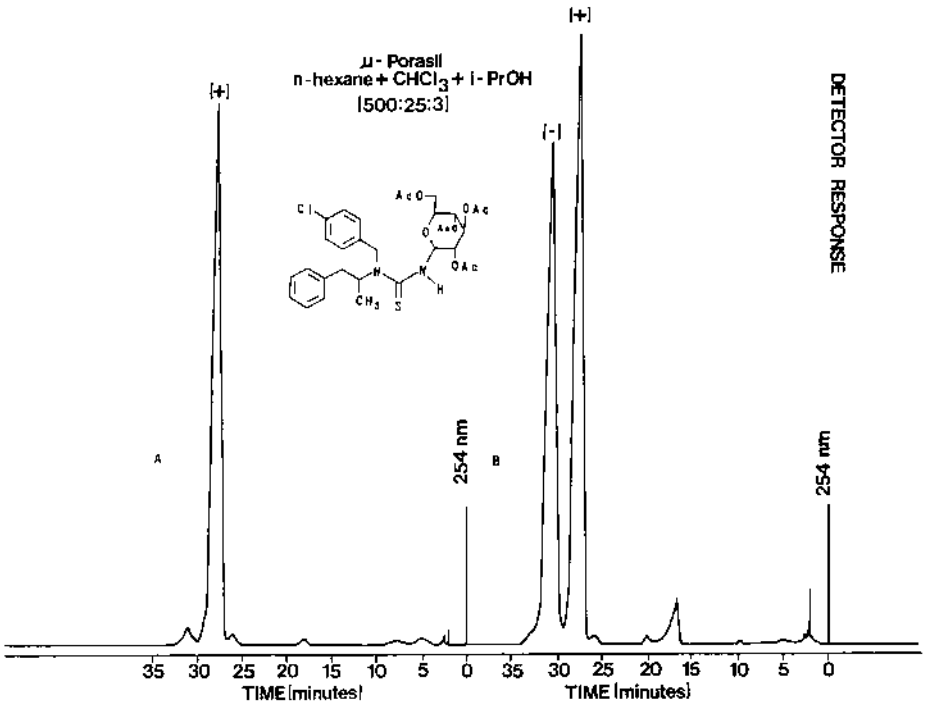

Figure 4. Normal-phase liquid chromatographic separation of GITC-derivatized enantiomers of N-(4-chlorobenzyl)-α-methylphenethylamine. A = (+)-enantiomer; B = racemic mixture.

of n-hexane, chloroform and isopropanol (500:25:3). Figures 2 - 4 show the GITC-derivatized (+)-isomers (Figures 2A, 3A and 4A) and the racemic mixtures (Figures 2B, 3B and 4B) for the 2-, 3- and 4-regiosiomeric N-(chlorobenzyl)-α-methylphenethylamines. In each case the A-chromatogram shows the regioisomer prepared from (+)-amphetamine to be predominately the (+)-enantiomer with perhaps a trace amount of the other enantiomer. This small amount of the other enantiomer may be due to enantiomeric contamination

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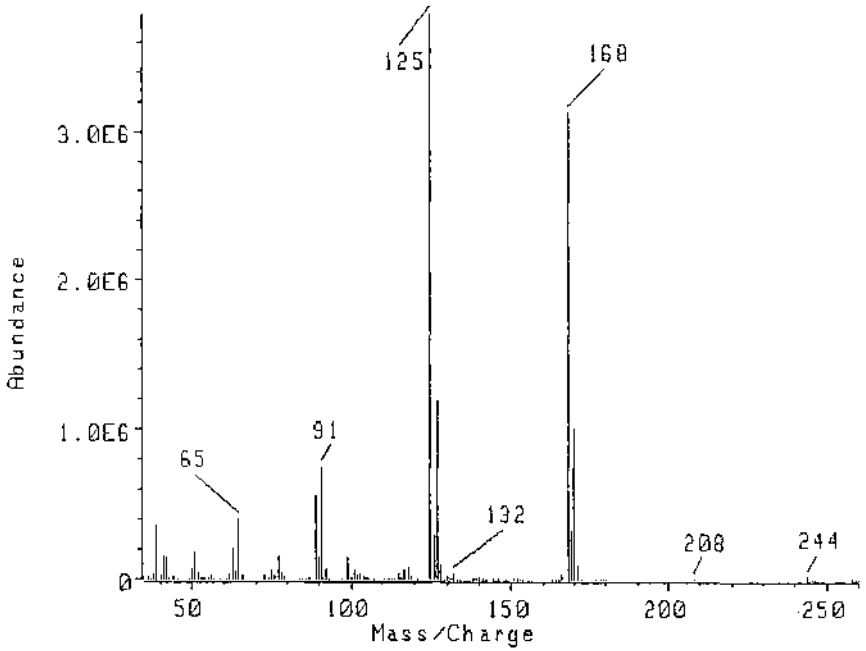

Figure 5. Mass spectrum of N-(2-chlorobenzyl)-α-methylphenethylamine.

of either the original amphetamine starting material or the GITC derivatizing reagent. The goal in most forensic analyses is not to determine the exact quantitative enantiomeric composition of a sample, but whether it consists primarily of one enantiomer or is a racemic mixture. Thus the presence of small amounts of the other enantiomers does not pose a significant problem in these analyses. In Figures 2B, 3B and 4B, the racemic amines are separated with near baseline resolution in each case. The (+)-enantiomer has the lower capacity factor in each case. These GITC-amine derivatives were not well resolved by reversed-phase methods. The combined use of derivatization methods (both PIT

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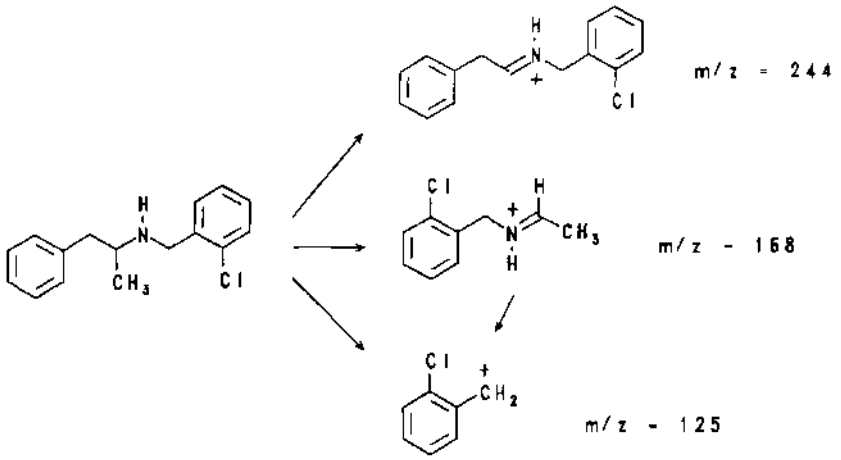

Scheme II. Mass spectral fragmentation pattern for the N-(chlorobenzyl)-α-methylphenethylamines.

and GITC) allows for the analysis of N-(chlorobenzyl)-α-methylphenethylamines for both positional and enantiomeric isomerism.

The mass spectrum of clobenzorex (the d-(+)-2-chloro regioisomer) is shown in Figure 5. The mass spectra of all isomers were identical since fragmentation is generally unaffected by such isomerism. The mass spectra were obtained in a GC-MS system using an OV-1 column with temperature programming. The base peak in the EI spectrum occurs at m/z 125 and is likely the chlorobenzyl fragment as shown in Scheme II. The other major fragment in the mass spectrum occurs from the loss of the unsubstituted benzyl radical from the alpha-cleavage reaction of the amino moiety. Thus, the mass spectrum confirms the N-(chlorobenzyl)-amphetamine structure, while the HPLC methods determine the position of the chloro substituent and the enantiomeric composition of the sample.

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Artículo original

Importancia de la preparación de la muestra en el estudio del metabolismo del clobenzorex

Importance of samples preparation in the study of clobenzorex's metabolism

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RESUMEN

Introducción: La selección del ensayo para estudiar el metabolismo de una sustancia depende de sus reacciones metabólicas, lo que puede influir positivamente en la selectividad del método y en la recuperación de los analitos, entre otros.

Objetivo: Definir la influencia de la preparación de la muestra en el estudio del metabolismo del clobenzorex en las condiciones de trabajo del Laboratorio Antidoping de La Habana.

Métodos: Se colectaron muestras de orina antes y después de la administración de una dosis de clobenzorex a un voluntario supuestamente sano. Se aplicaron tres ensayos A, B y C diseñados para la detección de metabolitos de fase I y de fase II.

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El método de extracción fue líquido-líquido y la diferencia entre los métodos fue la formación de derivados y la hidrólisis enzimática. La técnica instrumental empleada fue la cromatografía de gases acoplada a espectrometría de masas y el modo de adquisición de datos fue SCAN desde 50 a 600 Da.

Resultados: La detección de los metabolitos del clobenzorex más volátiles se vio afectada por la manipulación de la muestra, mientras que los hidroxilados (de fase I) necesitaron de una hidrólisis para ser detectados. Con la aplicación del método A se identificaron 3 compuestos, 5 con la aplicación del método B y con la aplicación del método C se identificaron los 8 metabolitos. A las 24 horas después de administrado el fármaco se pudieron detectar 6 de estos compuestos por el método C.

Conclusiones: La aplicación de los métodos adecuados de preparación de muestras, no solo para detectar el dopaje en atletas, sino también en el estudio del metabolismo de fármacos eliminados en la orina, define la cantidad y la calidad de la información obtenida. Bajo las condiciones del Laboratorio Antidoping de La Habana, una mejor detección del clobenzorex y sus metabolitos requiere de un paso de hidrólisis enzimática y uno de formación de derivados, en aras de obtener analitos con buenas condiciones cromatográficas al ser analizadas por la técnica de CG-EM.

Palabras clave: clobenzorex; metabolismo; hidrólisis; formación de derivados; cromatografía de gases; espectrometría de masas.

ABSTRACT

Introduction: The selection of the trial to study the metabolism of a substance depends on its metabolic reactions, which can positively influence the selectivity of the method and the collection of analytes, among others.

Objective: Define the influence of sample preparation in the study of clobenzorex metabolism in the conditions of the Anti-Doping Laboratory in Havana.

Methods: Urine samples were collected before and after the administration of a dose of clobenzorex to a supposedly healthy volunteer. Three trials (A, B and C)

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designed for the detection of Phase I and Phase II metabolites were applied. The extraction method was liquid-liquid and the difference between the methods was the derivatives formation and enzymatic hydrolysis. The instrumental technique used was gas chromatography coupled to mass spectrometry, and the data collection mode was SCAN from 50 to 600 Da.

Results: The detection of the most volatile clobenzorex's metabolites was affected by the manipulation of the sample, while the hydroxylated ones (phase I) required hydrolysis to be detected. With the application of method A, 3 compounds were identified, 5 with the application of method B and with the application of method C, 8 metabolites were identified. At 24 hours after administration of the drug, 6 of these compounds could be detected by method C.

Conclusions: The application of appropriate sample preparation methods, not only to detect doping in athletes, but also in the study of the metabolism of drugs eliminated in urine, defines the quantity and quality of the information obtained. Under the conditions of the Anti-Doping Laboratory of Havana, better detection of clobenzorex and its metabolites requires a step of enzymatic hydrolysis and one of derivative formation, in order to obtain analytes with good chromatographic conditions when analyzed by the CG-EM technique.

Keywords: Clobenzorex; metabolism; hydrolysis; derivatives formation; gas chromatography; mass spectrometry.

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Introducción

Uno de los retos de la analítica del dopaje es el conocimiento del metabolismo de las sustancias incluidas en la Lista de sustancias prohibidas para los atletas y que emite y actualiza la Agencia Mundial Antidopaje (AMA), con frecuencia anual.[1]

El conocimiento de las estructuras químicas, de las probables rutas metabólicas y los metabolitos formados permite determinar inequívocamente cuál fue la

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sustancia administrada, siempre que se seleccione adecuadamente la matriz y la técnica instrumental para su detección e identificación.

Aún no existe una técnica instrumental universal capaz de separar, detectar, cuantificar o identificar inequívocamente todos los analitos presentes en una matriz tan compleja como la orina. Las técnicas empleadas hasta el momento oscilan desde una simple dilución de la matriz (técnica *dilute and shoot* empleada en cromatografía líquida (CL) acoplada a espectrometría de masas (EM)[2,3] hasta las técnicas más complicadas que incluyen extracción en fase sólida o formación de derivados, entre otras.[4,5,6]

El conjunto de técnicas empleadas para extraer de la matriz analitos de interés y adecuarlos para su análisis instrumental se conoce ampliamente en el ámbito del dopaje como "preparación de la muestra". Lo que constituye uno de los pasos más importantes en la cadena analítica y puede ocupar del 60 % al 85 % del tiempo total de análisis. Del éxito de esta etapa dependerán parámetros analíticos tan importantes como la eficiencia de recuperación de analitos, la selectividad del método, el tiempo requerido para el análisis y su costo, entre otros.

La preparación de la muestra debe ser capaz de garantizar un extracto que sea compatible con el instrumento. Frecuentemente este hecho se menosprecia y crea problemas analíticos, generando los llamados *falsos negativos* donde no se detectan adecuadamente los analitos presentes en la matriz, o los *falsos positivos* donde se confunden los analitos de interés con señales interferentes producto de una extracción inadecuada de la matriz.

Dentro de las sustancias prohibidas por la AMA se encuentran los estimulantes del sistema nervioso central (SNC), varios de ellos con propiedades anorexígenas. La mayoría de estos compuestos muestran similitudes en sus propiedades químicas y físicas donde la presencia de un grupo amino le concede a la molécula propiedades básicas con un pKa entre 7-10. Son compuestos altamente volátiles y su masa molecular es menor de 350 Da, lo que hace factible su detección por la técnica de cromatografía de gases (CG). Si bien en la última década, la CL acoplada a la EM ha ganado espacio por las ventajas que ofrece, la CG acoplada a EM (CG-EM) aún ocupa un lugar predominante en los estudios metabólicos.[7,8,9,10,11]

Los estimulantes se pueden excretar en la orina en su forma intacta (droga madre administrada), pueden sufrir reacciones de desaminación, hidroxilación y redox entre otras reacciones de fase I. Además, pueden ser excretados como metabolitos de fase II conjugados con grupos sulfatos o glucurónido, fundamentalmente. El

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estudio del metabolismo de los estimulantes a partir de su excreción en orina se focaliza en la búsqueda de metabolitos en ambas fases.[12,13,14,15]

El diseño de un estudio enfocado en la detección de metabolitos de fase II mediante el uso de la técnica CG-EM debe incluir un paso de hidrólisis que separe el analito de su conjugado. De otra forma se intenta detectar un analito, generalmente de pobres propiedades cromatográficas, cuya masa es superior a la capacidad analítica del instrumento. La hidrólisis puede ser química o enzimática, la última es la preferida por ser altamente selectiva y evitar la formación de productos de degradación o la degradación del propio analito de interés. Además, debe tenerse en consideración la incompatibilidad de la columna cromatográfica capilar con matrices acuosas.[16,17,18]

La hidrólisis enzimática con extractos de *Helix pomatia* (con acción glucuronidasa y arilsulfatasa) se considera una opción adecuada para escindir complejos formados por enlaces con glucurónido y sulfatos. Sin embargo, su eficacia está influenciada por factores como el tiempo, temperatura y pH de incubación, así como la presencia de sales inorgánicas. Una vez que se completa la hidrólisis, el siguiente paso es lograr la separación de estos metabolitos (libres de su conjugado) de la matriz acuosa. En este caso es común realizar una extracción líquido-líquido donde el disolvente orgánico y el pH de extracción es un punto importante.[12,19,20,21]

Una vez que esté en fase orgánica, ya es factible hacer el análisis en el instrumento CG-EM, pero en aras de favorecer la detección, usualmente, se reduce la cantidad de disolvente por evaporación o se forman derivados de los analitos de interés. En este punto de la preparación de la muestra, debe tenerse en cuenta que los estimulantes son moléculas de bajo peso molecular, altamente volátiles e inestables. Generalmente poseen funciones químicas con hidrógenos activos tales como hidroxilos (-OH), aminas (-NH) y carboxilos (-COOH) que tienden a formar enlaces intermoleculares que afectan la volatilidad y tienden a interactuar con la fase estacionaria de las columnas cromatográficas, lo que conlleva a una disminución de la sensibilidad del método.[16,19,22]

Con el objetivo de minimizar estos efectos es usual favorecer las reacciones de formación de derivados. Es una reacción que modifica la funcionabilidad del analito brindando una mayor volatilidad, mayor estabilidad a altas temperaturas (como las que se encuentran en el inyector) y mejor respuesta en el detector. Se obtienen señales cromatográficas más simétricas, una mayor resolución entre picos cercanos y espectros de masas con mayor información sobre la molécula. La

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sililación es el método más frecuente de formación de derivados y consiste en la sustitución de un hidrógeno activo en la molécula (-OH o -NH) por un grupo trimetilsilil (TMS).[16,19,22]

El clobenzorex [(+)-N-(2-clorobencil)-1-fenilpropan-2-amina] es un fármaco anorexígeno, resultado de la modificación estructural de la anfetamina con la presencia de un sustituyente voluminoso en el grupo amino como es el clorobencilo. Pertenece al grupo de las feniletilaminas, es ópticamente activo y posee una masa molecular de 259 Da. Es una amina simpaticomimética que actúa sobre el núcleo ventrolateral del tálamo. Incrementa la secreción de norepinefrina y dopamina y disminuye su recaptura en las terminaciones nerviosas presinápticas.[14,15,23]

La unión a proteínas plasmáticas es escasa (15 %-30 %) pero muestra una rápida distribución tisular. El tiempo de vida media del clobenzorex es variable y puede oscilar en el intervalo de 1-17 horas. Se ha descrito que la dextroanfetamina es su metabolito mayoritario y que puede ser detectado en orina hasta 116 horas después de la administración de 30 mg del fármaco, con una concentración plasmática de 2,47 µg/mL.[14,15,24]

La biotransformación del clobenzorex transcurre por 2 vías metabólicas de fase I: (1) N-dealquilación mediante la actividad de la familia de los citocromos P450 que promueve la formación de anfetamina a partir de la molécula de clobenzorex y (2) reacciones de hidroxilación que origina al 4-hidroxiclobenzorex, dihidroxiclobenzorex y *p*-hidroxi-anfetamina. Cerca de un 40 % se excreta en forma de conjugados glucurónidos, el 1 % en forma de hipuratos, el 5 % en forma de anfetaminas y el 3 % como *p*-hidroxianfetamina. Se han descrito otros metabolitos de fase I. En el contexto de la analítica del dopaje se considera como metabolito marcador el 4-hidroxiclobenzorex y solo es detectable hasta 34 horas a partir de la administración del fármaco.[14,15,25] La figura 1 muestra las estructuras químicas de las sustancias relacionadas con el metabolismo del clobenzorex que fueron incluidas en el estudio.

El objetivo principal de la presente investigación es definir la influencia de la preparación de la muestra en el estudio del metabolismo del clobenzorex en las condiciones de trabajo del Laboratorio Antidoping de La Habana.

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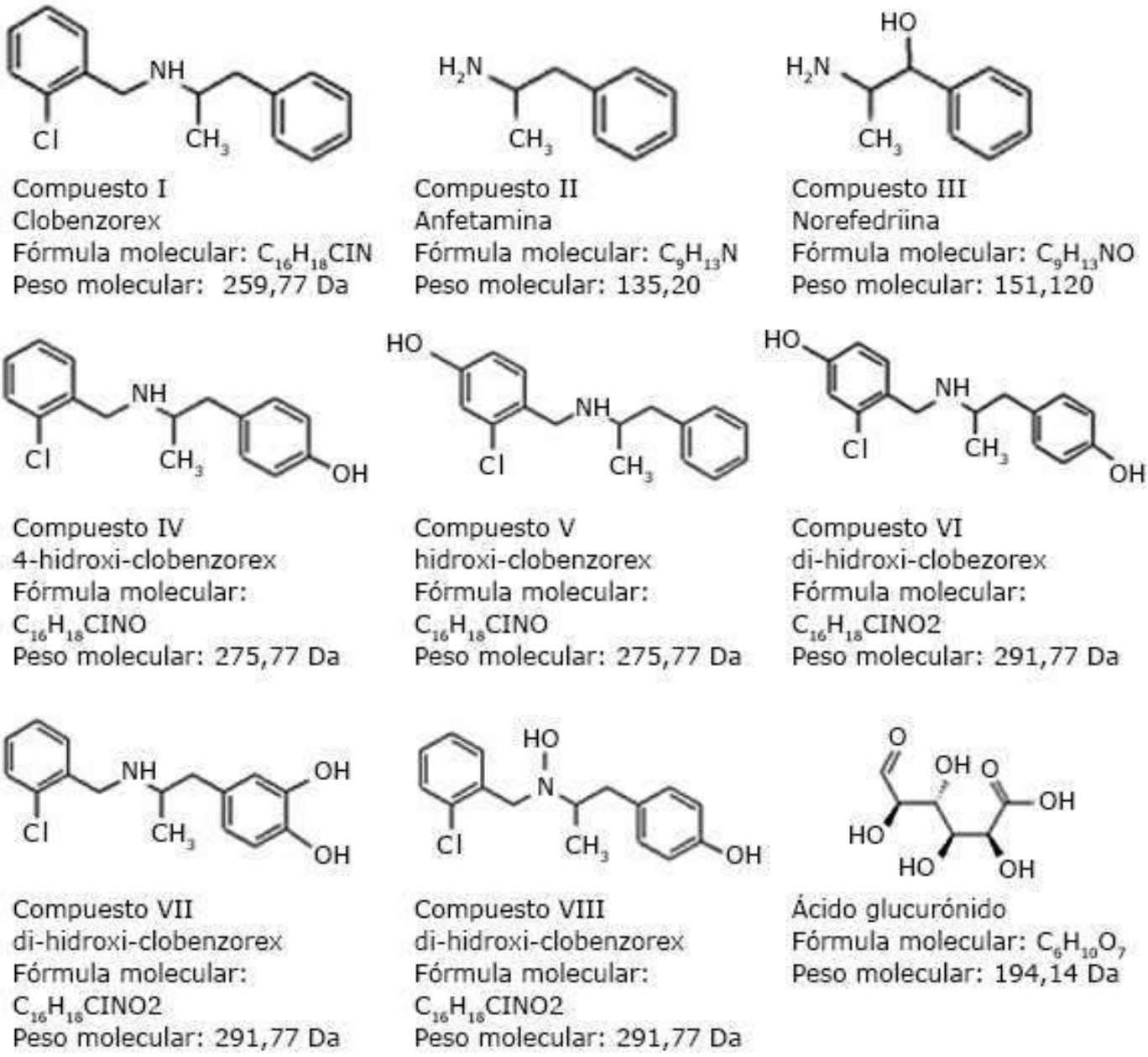

Fuente: Hemmersbach P. History of mass spectrometry at the Olympic Games. J Mass Spectrom. 2008,43:839-53.

Fig. 1 - Estructuras químicas del clobenzorex y de los metabolitos considerados en el estudio (compuestos I al VIII) y del ácido glucurónido.

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Métodos

Reactivos: Tert-butil metileter pureza > 99 %, para análisis (Merck, Alemania); metanol pureza 99,93 %, grado espectroscópico (Merck, Alemania); acetato de etilo pureza 99,8 % para cromatografía líquida LiChrosolv®, (Merck (Alemania); hexano pureza 99 % (Merck, Alemania); sulfato de sodio anhidro, pureza > 99,7%, para análisis (Merck, Alemania); hidróxido de amonio (Sigma-Aldrich, Alemania); enzima ß-glucuronidasa/arilsulfatasa; de *Helix Pomatia* (Roche, EE. UU.); N-metil-N-trimetilsililtrifluoracetamida (MSTFA, derivados TMS), para síntesis (Merck, Alemania); N-metil-bistrifluoracetamida (MBTFA, derivados TFA), pureza > 97 % (Sigma-Aldrich, Alemania).

Material de referencia usado: anfetamina (ampolleta conteniendo una disolución de 1 mg/mL en metanol) y norefedrina clorhidrato, ambos obtenidos de Sigma-Aldrich. El clobenzorex fue gentilmente donado por el Laboratorio Acreditado de Roma, Italia.

Muestras empleadas en el estudio: Las muestras de orina empleadas en el estudio provienen de un individuo supuestamente sano al que se administró una dosis única de clobenzorex por vía oral. Al voluntario (sexo femenino, 22 años, de 54 kg de peso corporal y actividad física moderada) se le administró una dosis única de una tableta de 30 mg de clobenzorex con 240 mL de agua. Las muestras de orina se colectaron antes de la administración del fármaco y posterior a la administración se colectaron cada tres horas hasta las 24 horas (0, 3, 6, 9, 12, 15, 18, 21 y 24 h).

Consideraciones éticas: El estudio fue aprobado por el Comité de ética del Instituto de Medicina Deportiva. En el experimento se siguió lo establecido en la Declaración de Helsinki y las recomendaciones de la Organización Mundial de la Salud (OMS) para la investigación en humanos.[26]

Métodos de preparación de las muestras de orina para su análisis instrumental: Se establecieron tres métodos de trabajo para obtener información acerca de los metabolitos urinarios del clobenzorex y observar su influencia en su detectabilidad. La figura 2 describe la preparación de la muestra en cada método de trabajo. El método A debe detectar los metabolitos de fase I que se excretan en forma libre de conjugados. El método B debe detectar los mismos metabolitos del método A, pero con una mayor sensibilidad instrumental, debido a la formación de derivados. El método C debe mostrar la presencia de los metabolitos observados en el método B y adicionalmente los metabolitos de fase II.

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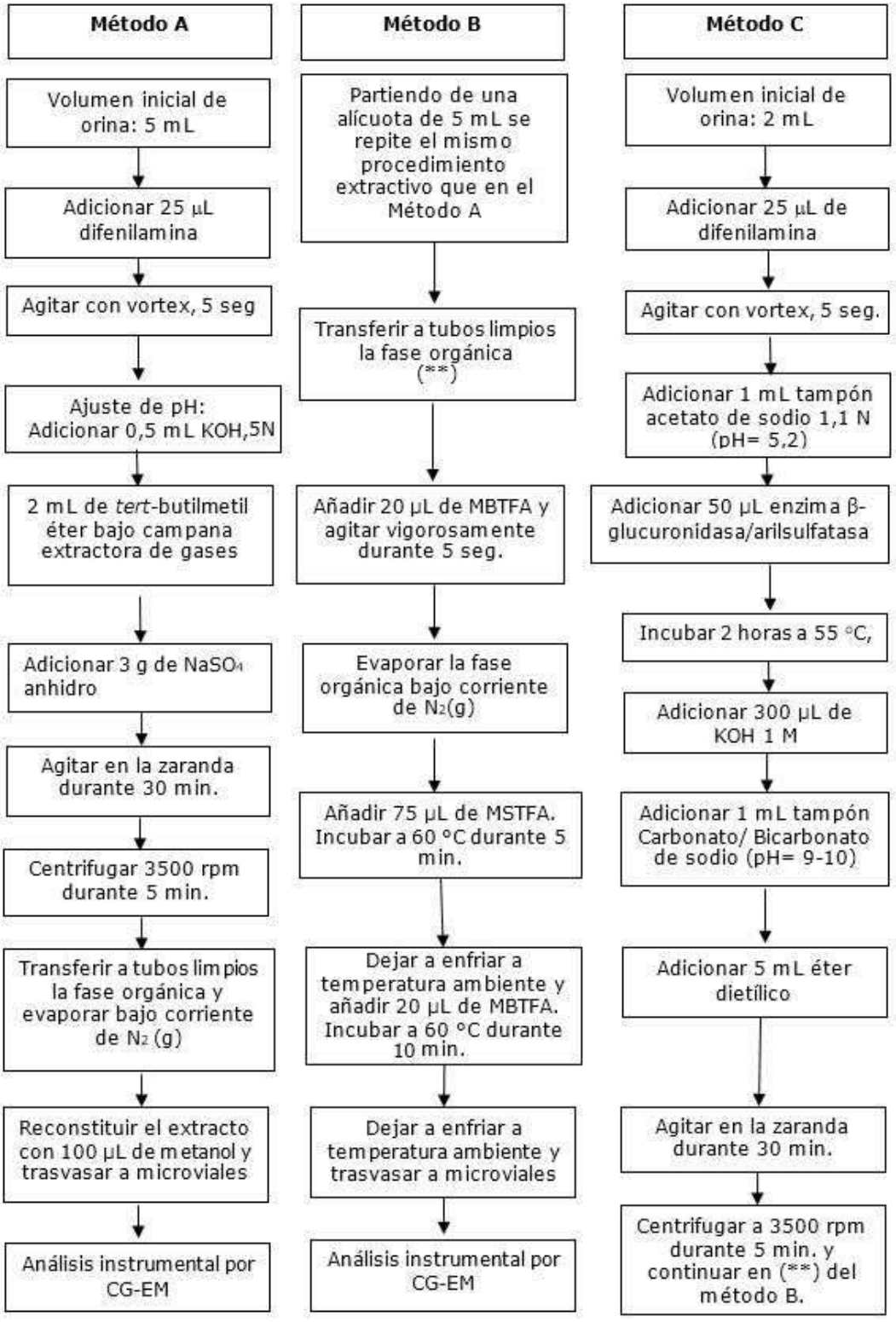
Método A
Volumen inicial de orina: 5 mL
Adicionar 25 µL difenilamina
Agitar con vortex, 5 seg
Ajuste de pH: Adicionar 0,5 mL KOH,5N
2 mL de tert-butilmetil éter bajo campana extractora de gases
Adicionar 3 g de NaSO4 anhidro
Agitar en la zaranda durante 30 min.
Centrifugar 3500 rpm durante 5 min.
Transferir a tubos limpios la fase orgánica y evaporar bajo corriente de N2 (g)
Reconstituir el extracto con 100 µL de metanol y trasvasar a microviales
Análisis instrumental por CG-EM
Método B
Partiendo de una alícuota de 5 mL se repite el mismo procedimiento extractivo que en el Método A
Transferir a tubos limpios la fase orgánica (**)
Añadir 20 µL de MBTFA y agitar vigorosamente durante 5 seg.
Evaporar la fase orgánica bajo corriente de N2(g)
Añadir 75 µL de MSTFA. Incubar a 60 °C durante 5 min.
Dejar a enfriar a temperatura ambiente y añadir 20 µL de MBTFA. Incubar a 60 °C durante 10 min.
Dejar a enfriar a temperatura ambiente y trasvasar a microviales
Análisis instrumental por CG-EM
Método C
Volumen inicial de orina: 2 mL
Adicionar 25 µL de difenilamina
Agitar con vortex, 5 seg.
Adicionar 1 mL tampón acetato de sodio 1,1 N (pH= 5,2)
Adicionar 50 µL enzima β-glucuronidasa/arilsulfatasa
Incubar 2 horas a 55 °C,
Adicionar 300 µL de KOH 1 M
Adicionar 1 mL tampón Carbonato/ Bicarbonato de sodio (pH= 9-10)
Adicionar 5 mL éter dietílico
Agitar en la zaranda durante 30 min.
Centrifugar a 3500 rpm durante 5 min. y continuar en (**) del método B.

Fig. 2 - Flujo de trabajo y descripción de los tres métodos aplicados a las muestras de orina.

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En cada lote de análisis se incluyeron controles positivos que consistieron en orinas contaminadas con clobenzorex, anfetamina y norefedrina en concentración de 200 ng/mL. A los que se les aplicaron los métodos de trabajo correspondientes. El patrón interno (P.I.) fue difenilamina (1 mg/mL).

Equipamiento: cromatógrafo de gases Hewlett Packard 6890 *plus*, con inyector automático Agilent 7683 acoplado a espectrómetro de masas Hewlett Packard 5973 (CG-EM). Columna capilar HP-Ultra 2, fase estacionaria fenil metil silicona 5 %, 12 m de longitud, 0,20 mm de diámetro interno y 0,33 µm de espesor de película. El volumen de inyección fue de 3 µL en modo split (relación 1:10) y el gas portador fue helio a un flujo de 1 mL/min. La temperatura del inyector fue de 250 ºC para el método A y 280 ºC para los métodos B y C. La temperatura del detector fue de 280 ºC y el modo de adquisición de datos fue en modo SCAN en un rango de masas de 50 Da a 600 Da.

Evaluación de los datos: Los cromatogramas y espectros de masas se obtuvieron con el software Enhanced Chemstation MSD E.02.01.1177 Copyright© 1989-2010 Agilent Technologies Inc. A partir del espectro de masas de cada metabolito, se obtuvo el área bajo la curva del ion base. La estimación de las concentraciones de cada analito en cada muestra se realizó a partir de la relación de áreas con el patrón interno. El área bajo la curva de cada metabolito se corrigió por la gravedad específica de la orina (g.e) para normalizarlas al valor de 1,020. Para ello se multiplicaron por un factor de corrección calculado según: Fc = (g.e. muestra -1)/(1,020-1).[27]

Resultados

Los resultados obtenidos luego de aplicar el método A muestran la presencia de clobenzorex, anfetamina y norefedrina identificados en la tabla como los compuestos I, II, y III, respectivamente.

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Tabla - Resumen de los resultados obtenidos en el estudio del metabolismo del clobenzorex mediante la aplicación de tres métodos diferentes de preparación de la muestra de orina

ID	Descripción	Parámetro	Método A	Método B	Método C
I	Clobenzorex Fármaco administrado	TR (min)	7,3	7,5	7,5
		C_{max} (ng/mL)	7,2	1,5	62
		t_{max} (h)	3	3	3
		Conc 24h (ng/mL)	0	0	0
II	Anfetamina (metabolito)	TR (min)	2,3	3,0	3,0
		C_{max} (ng/mL)	305	2265	1144
		t_{max} (h)	6	6	6
		Conc 24h (ng/mL)	191	455	207
III	Norefedrina (metabolito)	TR (min)	3,6	4,1	4,1
		C_{max} (ng/mL)	112	2025	1350
		t_{max} (h)	6	6	6
		Conc 24h (ng/mL)	31	485	323
IV	Metabolito hidroxilado en la posición *p*- del clorobencilo	TR (min)	8,1	8,6	8,6
		C_{max} (ng/mL)	---	11	2630
		t_{max} (h)	---	3	3
		Conc 24h (ng/mL)	---	0	30
V	Metabolito hidroxilado en la posición *p*- del grupo bencilo	TR (min)	---	8,8	8,8
		C_{max}/(ng/mL)	---	4	9352
		t_{max} (h)	---	3	3
		Conc 24h (ng/mL)	---	0	345
VI	Metabolito hidroxilado en la posición *p*- del grupo bencilo y clorobencilo	TR (min)	---	---	9,9
		C_{max} (ng/mL)	---	---	476
		t_{max} (h)	---	---	3
		Conc 24h (ng/mL)	---	---	27
VII	Metabolito dihidroxilado en las posiciones *o*- y *p*- del grupo bencilo	TR (min)	---	---	9,6
		C_{max} (ng/mL)	---	---	4237
		t_{max} (h)	---	---	3
		Conc 24h (ng/mL)	---	---	41
VIII	Metabolito dihidroxilado en la posición *p*- del grupo bencilo y en el grupo amino	TR (min)	---	---	9,3
		C_{max} (ng/mL)	---	---	67
		t_{max} (h)	---	---	3
		Conc 24h (ng/mL)	---	---	0

TR: tiempo de retención; min.: minutos; Cmax : concentración máxima observada en orina; ng/mL: nanogramos por mililitro de orina; tmax: tiempo al que se encuentra la concentración máxima; h: horas; Conc 24 h: concentración observada en orina a las 24 horas después de la administración de clobenzorex.

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Con la aplicación del método A, la concentración máxima de anfetamina en las muestras colectadas, fue de 305 ng/mL en el intervalo de 3-5 horas después de la administración del fármaco. Sin embargo, con la aplicación de los métodos B y C, la concentración máxima observada fue de 2265 ng/mL y 1144 ng/mL, respectivamente.

Los compuestos IV y V (Tabla) son de fase II, por lo que no fue posible su detección por la aplicación del método A. La presencia del grupo glucurónido otorga a la molécula una alta solubilidad en medio acuoso y por tanto no es posible extraerlo en el disolvente orgánico. Mediante la aplicación de este método, los metabolitos IV y V permanecen en la orina (fase desechada).

Al evaluar los metabolitos por el método B se detectaron concentraciones cercanas a los 10 ng/mL a las 6 h, lo cual es coherente con otros autores,[15,28] alrededor del 1 % de los metabolitos hidroxilados de fase II no llegan a conjugarse y por tanto se pueden eliminar en la orina en su forma libre. En teoría, el 1 % detectado por el método B debería observarse igualmente por el método A, sin embargo, no ocurrió así. Este resultado pone de manifiesto la ventaja que ofrece la formación de derivados en la sensibilidad del ensayo.

El análisis de las muestras con el método B mostró concentraciones del compuesto IV cercano a los 11 ng/mL. Con la inclusión del paso de hidrólisis (método C) la concentración máxima de este compuesto se estimó en 2630 ng/mL.

El compuesto V, conocido en la literatura especializada como el 4-hidroxiclobenzorex, se pudo observar a las 3 h en una concentración máxima aproximada de 9350 ng/mL, con la aplicación del método C. En el intervalo de las 21 h -24 h la concentración estimada osciló entre 250 ng/mL -350 ng/mL confirmando, según lo descrito por otros autores, que es el metabolito de mayor importancia en el metabolismo del clobenzorex.[14] Al aplicar los métodos A y B no se observó señal correspondiente a los analitos identificados como VI y VII (Tabla) y solamente al aplicar el método C pudieron ser detectados.

El compuesto VI alcanzó su concentración máxima aproximada de 480 ng/mL en las primeras 5 h después de la administración de clobenzorex y, aún, al final del estudio, entre las 21 h-24 h, pudo ser detectado con concentraciones en el rango de 21 ng/mL-27 ng/mL. En el caso del compuesto VII, el comportamiento fue similar, sin embargo, los valores de concentración máxima alcanzaron los 4200 ng/mL en el mismo tiempo y en el intervalo de las 21 h-24 h la concentración se observó entre los 33 ng/mL-41 ng/mL.

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Luego de aplicar el método C, se observó una señal cromatográfica a los 9,3 minutos con un espectro de masas característico de estructuras con presencia de un átomo de cloro y con una curva de eliminación en concordancia con la administración oral (Fig. 3). Este compuesto fue identificado en la tabla como compuesto VIII y se detectó solamente durante las primeras 15 horas. La concentración máxima estimada fue de 67 ng/mL a las 3 h y durante el intervalo de las 6 h-15 h los valores no superaron los 10 ng/mL.

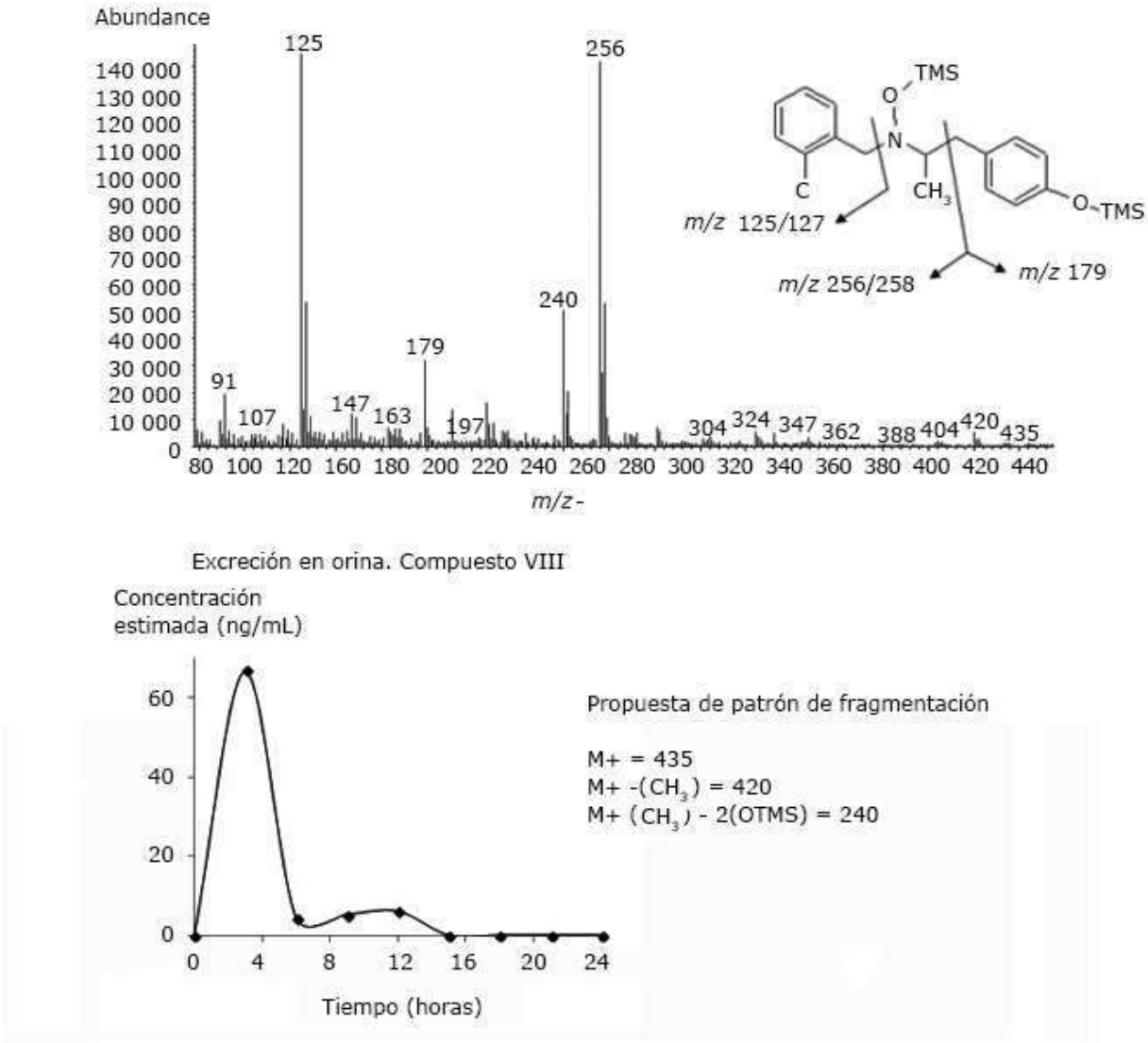

Fig. 3 - Espectro de masas, propuesta de fragmentación y curva de eliminación urinaria del compuesto identificado como VIII y que pudiera ser atribuible a la hidroxilación del grupo amino y el anillo bencilo.

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Discusión

Los principales metabolitos del clobenzorex de fase I y fase II están descritos en la literatura especializada, fundamentalmente en el ámbito de la analítica del dopaje. La importancia de este trabajo se basa en la selección de un método que permita enfocar el estudio de estos metabolitos del clobenzorex en las condiciones de trabajo del Laboratorio Antidoping de La Habana. El resultado final no solo es aplicable con propósitos de control antidopaje sino también con el objetivo de estudiar la farmacología del compuesto.

La reacción de formación de derivados justo antes de la evaporación del solvente orgánico (método B), otorgó a la molécula de anfetamina cierta estabilidad disminuyendo su volatilidad durante el paso de evaporación bajo la corriente de nitrógeno y mejoró considerablemente sus propiedades cromatográficas. El método C mostró niveles más bajos, probablemente debido a la manipulación de la muestra, por ejemplo, la hidrólisis que ocurre a 55 ºC durante 2 h y los ajustes de pH necesarios.

Metabolitos monohidroxilados

El compuesto identificado como IV (Tabla) con el grupo OH en la posición "*p*-" del clorobencilo, fue descrito anteriormente por *Maurer* y otros.[29] Al igual que el compuesto V (hidroxilado en el grupo bencilo), son metabolitos de fase II y se excretan en forma de conjugado con el ácido glucurónico.

La masa de ambos compuestos hidroxilados es de 275 Da y la masa del grupo glucurónido es de 194 Da, es decir, la masa de la molécula glucuronada sería de 451 Da, lo cual es detectable por el instrumento, pues el rango de masas de adquisición es de 50 Da a 600 Da. Pero la presencia de 4 grupos hidroxilo con hidrógenos activos le otorga a la molécula propiedades cromatográficas pobres, con picos deformados y colas que van en detrimento de una adecuada detección y resolución de analitos cercanos. La formación de derivados de estas moléculas podría ser la solución al problema cromatográfico y así, la masa total del compuesto glucuronado con los grupos trimetilsilil correspondientes sería de 811 Da. Esta masa se encuentra fuera del rango de medición del instrumento, por tanto, el metabolito no es detectable. Al introducir el paso de la hidrólisis se separa el grupo glucurónido de los analitos de interés, logrando así una mejora considerable de sus propiedades cromatográficas y una adecuada detección mediante el uso de la técnica CG-EM.

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La eliminación del metabolito identificado como IV fue rápida, pero incluso a las 24 horas (última muestra de orina colectada) pudo ser detectado en concentraciones cercanas a los 20 ng/mL. La presencia de un átomo de cloro en su molécula le otorga al espectro de masas características especiales, la relación señal: ruido es alta y la selectividad y sensibilidad del método se ven favorecidos.

Los resultados obtenidos para el metabolito V (el 4-hidroxiclobenzorex) corroboran lo descrito anteriormente respecto a la preparación de la muestra de orina basado en la hidrólisis enzimática seguido de la formación de derivados como pasos indispensables para la correcta detección de ambos metabolitos.[14,15]

Metabolitos polihidroxilados

Entre los metabolitos polihidroxilados del clobenzorex se encuentran descritos en la literatura el *p*-OH-bencilo- *p*-OH-clorobencilo (compuesto VI, Tabla) y el *o*, *p*-diOH-bencilo (compuesto VII, Tabla),[28] ambos identificados adecuadamente.

La estructura química del clobenzorex y sus posibilidades de biotransformación permiten la N-hidroxilación del grupo amino catalizada por una enzima de la familia citocromo P450 y, además, una hidroxilación del anillo bencilo. En este caso el metabolito debería conjugarse (fase II) antes de ser excretado en la orina. La figura 3 muestra el espectro de masas para el compuesto VIII y la propuesta del patrón de fragmentación. Se apreció el par de iones *m/z* 125/127 característico del clorobencilo y la existencia del fragmento *m/z* 256/258 que indica la probable presencia del grupo hidroxilo como sustituyente en el grupo amino. Además, el ión *m/z* 179 justifica la presencia de un grupo hidroxilo (trimetilsililado) en el anillo A, probablemente en posición "*para*-". La descripción de la estructura a partir de un espectro de masas es solamente una propuesta. Para la elucidación de la estructura química de nuevas sustancias se requiere de técnicas complementarias como la espectroscopia infrarroja (IR) o la resonancia magnética nuclear (RMN), entre otras.

Es importante tener en cuenta, a pesar de las ventajas conocidas, que la formación de derivados posee desventajas que, de no tenerse en consideración, podrían influir en la interpretación de los resultados. Con los reactivos empleados en la formación de derivados se espera obtener derivados TFA-TMS, sin embargo, no puede excluirse la formación, aunque en menor proporción, de derivados TMS-TMS. La formación de derivados mixtos afecta la cuantificación de sustancias y es por eso que en el presente trabajo se describen solamente concentraciones estimadas y no exactas.

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Comparación de los tres métodos de trabajo

La tabla muestra la comparación de los tres métodos de trabajo A, B y C. Los resultados obtenidos demuestran que con la aplicación de los métodos A y B el Laboratorio Antidoping de La Habana solo es capaz de detectar la anfetamina, la norefedrina y el propio clobenzorex (droga madre). En el caso de este último solamente se observaron valores alrededor de 10 ng/mL hasta las 10 h posadministración, mientras que para la anfetamina y la norefedrina se observaron mayores concentraciones, aún a las 24 h, siempre en el orden de los ng/mL de orina. Es importante decir, que siendo la anfetamina y la norefedrina metabolitos comunes de otras sustancias prohibidas (por ejemplo, etilanfetamina, famprofazona, fencamina, fenetilina), la aplicación de estos dos métodos por sí solos reducen la capacidad analítica del laboratorio para detectar el consumo de clobenzorex con propósitos de dopaje.

Mientras que el método B resultó ser el método óptimo para la detección de la anfetamina y la norefedrina por mejorar significativamente sus condiciones cromatográficas, el método C demostró lo contrario, probablemente por la mayor manipulación de la muestra. Sin embargo, los metabolitos hidroxilados (monohidroxilados y polihidroxilados) excretados como metabolitos de fase II brindan mayor información diagnóstica y su detección es indudable, solamente si se aplica el método C.

Teniendo en cuenta la estructura química del clobenzorex no se debe descartar una probable excreción de metabolitos conjugados con grupos sulfatos. Esta es una vía importante de la biotransformación de grupos fenólicos y de grupos hidroxi-alifáticos, así como de ciertos neurotransmisores, ácidos biliares e hidroxilaminas orgánicas. Las enzimas responsables de la conjugación con sulfato son las sulfotransferasas. Estas enzimas catalizan la sulfatación de los xenobióticos con carácter fenólico, así como catecolaminas, estrógenos y esteroides.[1,2,30] Con el uso de la enzima ß-glucuronidasa-arilsulfatasa contenida en extractos de *H. pomatia* y empleada en el método C, no es posible diferenciar entre un metabolito de fase II conjugado con sulfatos o con glucurónido.

En síntesis, se puede plantear que el mejor método para la detección de anfetamina y norefedrina es el método B. Los metabolitos hidroxilados de fase I que son eliminados en forma de conjugados (fase II) resultan ser los de mayor valor diagnóstico y requieren de un paso de hidrólisis, por ello el método C resulta ser el adecuado para su detección.

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Por lo que se concluye que la aplicación de los métodos adecuados de preparación de muestras, no solo para detectar el dopaje en atletas, sino también en el estudio del metabolismo de fármacos eliminados en la orina, define la cantidad y la calidad de la información obtenida. Bajo las condiciones del Laboratorio Antidoping de La Habana, se pudo determinar que una mejor detección del clobenzorex y sus metabolitos requiere de un paso de hidrólisis enzimática y uno de formación de derivados, en aras de obtener analitos con buenas condiciones cromatográficas al ser analizadas por la técnica de CG-EM.

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Scientific Research

Comparison on the Pharmacokinetics and Weight Reduction of Clobenzorex Slow Release and Immediate Release Formulations in Obese Patients

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ABSTRACT

Clobenzorex is an anorexigenic drug that is widely used in Mexico for the treatment of obesity, since it helps to reduce body weight. This drug is available as immediate release capsules. To improve compliance to treatment, it was developed a new slow release formulation. In order to establish its usefulness, oral pharmacokinetics and weight reduction of slow release and immediate release formulations of clobenzorex in obese patients were compared. Sixty patients with a BMI higher than 27 kg/m^2 were included in the study. Two groups of 30 patients were formed, one of them received 30 mg immediate release formulation b.i.d. and the other group received one 60 mg slow release formulation once a day, since under this scheme these formulations are prescribed. Blood samples were obtained at selected times during the first day and once weekly during 4 weeks. After the last dose, samples were obtained at selected times during 48 h. Plasma levels were determined by HPLC-MS/MS and pharmacokinetic parameters were obtained. Reduction in C_{max} due to increased t_{max}, as well as, increased half-life were observed with the slow release formulation in comparison with immediate release formulation. Although lower plasma levels of clobenzorex were reached with the slow release formulation, reduction of body weight was similar with both products. Based on the results, it was concluded that slow release formulation of clobenzorex is an adequate formulation of clobenzorex, since pharmacokinetics and effects observed are compatible with a once a day administration.

Keywords: Clobenzorex; Pharmacokinetics; Slow Release Formulation

1. Introduction

Clobenzorex is a sympathomymethic amine with low addictive activity and with anorexygenic activity that is widely used in Mexico for the treatment of obesity. It is recommended for patients with BMI higher than 30 kg/m^2 who did not respond to diet and exercise. Clobenzorex produces its anorexygenic activity by increasing noradrenaline levels; this noradrenaline reduces appetite by activating alpha-4 and beta-1 adrenergic receptors in hypothalamus [1,2].

Clobenzorex has a pKa of 8.8 to 10.4 and after oral administration, clobenzorex is well absorbed in gastrointestinal tract, reaching the maximal concentration in about 1 to 1.5 hours and plasma levels varies between 8 to 47 ng/ml [3,4]. Clobenzorex is widely distributed in tissues, in fact, levels reached in spinal cord fluid are similar to those reached in plasma. It is bound to plasma proteins in about 15% - 30% and is metabolized mainly to 4-hydroxyclobenzorex. Half-life of clobenzorex is widely variable, since values observed are from 1 to 17 hours.

Clobenzorex is eliminar palabra used as an immediate release formulation at dosage regimes of 30 mg b.i.d. [5], however, peak concentration reached may produce a high incidence of side effects and may increase the probability of drug abuse. In order to make the treatment more suitable and reducing both, side effects and probability of

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drug abuse, a slow release formulation was developed. The purpose of this study was to compare the pharmacokinetics and the reduction of body weight of these formulations in overweighted and obese patients under treatment for one month.

2. Subjects, Material and Methods

Sixty patients (45 women, 15 men) with body mass index higher than 27 kg/m^2 were included in the study. All subjects were evaluated by clinical history, medical examination and suitable laboratory tests, in order to discard any comorbility. All patients that met the inclusion criteria were randomly assigned to one of two groups of thirty subjects. Comparison of demographic data of treatment groups are given in **Table 1**. Group one received treatment with immediate release capsules of 30 mg b.i.d. for four weeks, whereas, group two received slow release tablet of 60 mg once a day for the same period. These treatment schemes were employed, since immediate release formulation was prescribe under these conditions and slow release formulation was intend to be used once a day. Once the subjects were assigned to the treatment group they were hospitalized to start the treatment with clobenzorex and after the first dose, blood samples were collected at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h. Plasma was obtained by centrifugation and stored frozen at −80˚C until analyzed. After obtaining the last blood sample, patients were provided of medication enough for a week and allowed to continue their normal activities. They were instructed to come back to the clinical facilities after a week for evaluation of anthropometric measurements and obtaining through plasma sample. This procedure was repeated on week 2, 3 and 4. After the last dose administration, subjects were maintained in the clinical facilities and blood samples were obtained at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24 and 48 h. Plasma was obtained and stored as described above. A final evaluation of anthropometric parameters was carried out.

2.1. Determination of Clobenzorex in Plasma

Plasma levels of clobenzorex were determined by a high-performance liquid chromatographic method coupled to MS/MS detection. Briefly, 0.5 ml were placed in 15 ml conical tubes and added with 12.5 ng of paroxetine as internal standard and 4 ml of methyl-tert-butyl ether. This mixture was agitated in a vortex mixer at maximal speed for 1 minute and centrifuged at 3500 rpm for 10 minutes. Upper layer was transferred to another tube and evaporated to dryness at 35˚C under nitrogen stream. Dry residue was redissolved in 200 µl of mobile phase (see under) and 10 µl aliquots were injected into the chromatographic system.

The chromatographic system was formed by an acquity ultra performance liquid chromatograph (Waters Assoc, Milford, MA, USA) coupled to a API-3200 mass/mass spectrometer (Applied Biosystems, Inc.). Separation of compounds was carried out using an acquity UPLC BEH Phenyl 100 mm length × 2.1 mm i.d. of 1.7 µm particle size column eluted with a mixture of methanol with 0.3% formic acid solution (75:25, v/v) at a flow rate of 0.2 ml/min. The sample were subsequently analyzed by mass spectrometry with an ESI source and then quantified by multiple reaction monitoring (MRM). The precursor-product ion combinations of m/z 260.1 → 91.1 and m/z 330.1 → 70.0 was used to quantify clobenzorex and internal standard, respectively. Under these conditions, clobenzorex retention time is 1.22 min and paroxetine is 1.29 min and no interferences occurs at this time. Typical chromatograms obtained with the employed method are shown in **Figure 1**.

Under these conditions, the method was linear in the range of 0.05 to 100 ng/ml, with an intra- and inter-day accuracy of 95.33% and the coefficient of variation was lower than 13%.

2.2. Pharmacokinetic and Statistical Analysis

Individual plasma level-time curves were constructed and maximal concentration (Cmax) and time to reach this

Table 1. Demographic data of subjects enrolled in the study of comparison of pharmacokinetics and body weight after administration of immediate release and slow release formulations of clobenzorex. Data are expressed as mean ± SD. No statistically significant difference in any parameter was obtained.

	Clobenzorex slow release formulation (60 mg) n = 30	Clobenzorex immediate release formulation (30 mg) n = 30
Age (years)	35.03 ± 8.97	33.97 ± 11.43
Sex (F/M)	23/7	22/8
Weight (kg)	90.51 ± 15.88	84.07 ± 15.51
Waist (cm)	105.9 ± 13.24	100.76 ± 12.18
Height (m)	1.642 ± 0.078	1.607 ± 0.090
BMI (kg/m^2)	33.47 ± 4.77	32.54 ± 4.08

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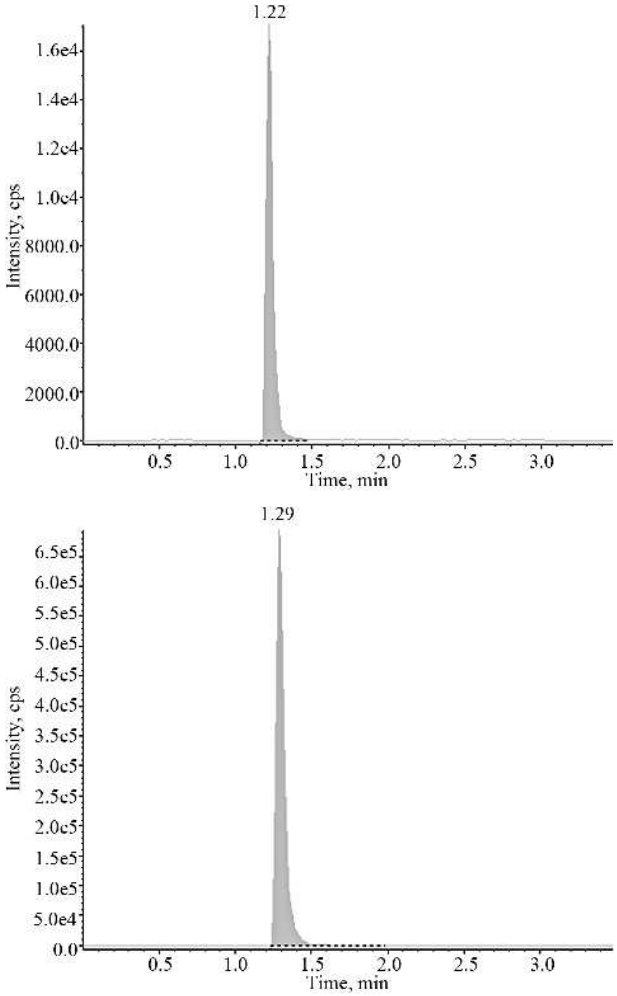

Figure 1. Typical chromatograms obtained after injection of the extract of a blank plasma spiked with both, 25 ng/ml clobenzorex (upper part) and 25 ng/ml of the internal standard (lower part).

maximum (Tmax) were directly obtained of such curves. Area under the plasma concentration against time curve until the last sampling time (AUClast) was obtained by the trapezoidal rule, extrapolation to infinity was carried out by dividing the last concentration observed by the elimination constant rate value [6]. Comparison of pharmacokinetic parameters was carried out by student-t-test. On the other hand, weight and body mass index reduction with the formulations tested were compared by analysis of variance followed by Tukey test.

3. Results

Figure 2 shows plasma level-time curves after the first administration of clobenzorex as a 30 mg immediate release capsule and a 60 mg slow release tablet. It can be seen that lower concentrations with the slow release formulation are reached, however, long lasting concentrations were observed with such formulation. Pharmacokinetic parameters obtained are shown in **Table 2**. It is important to note that although frequency of administration was different with the formulations tested, through levels are similar, as shown in **Table 3**.

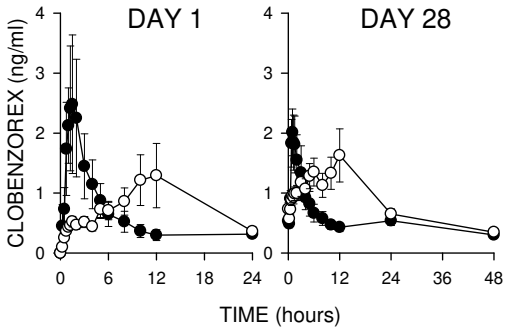

Figure 2. Plasma concentration against time curves after administration of clobenzorex 30 mg immediate release capsule b.i.d (dark circles) and 60 mg slow release tablet (open circles) after the first dose (left side) and after the day 28 (right side). Data are expressed as mean ± s.e.m. of 30 subjects per group.

Table 4 shows the comparison of the reduction of body weight and body mass index in the subjects submitted to both treatments. It is clearly shown that no statistically significant difference was observed in the rate of loss of weight during the treatment with both formulations. It is important to note that both treatments were well tolerated. Only 7 mild events were observed with immediate release formulation and 10 mild events with the slow release formulation, being the most frequent adverse events headache, dizziness and nausea.

4. Discussion

Pharmacokinetics and weight reduction of clobenzorex administered as 30 mg immediate release capsule b.i.d. and 60 mg slow release tablet once a day were compared. Although clobenzorex is one of the best seller drugs in Mexico, so far, no information about its pharmacokinetics in Mexicans is available.

When pharmacokinetics of the two formulations was compared, it was observed that through levels reached with both schemes are similar, but maximal concentration was reduced. This last was expected for a slow release formulation. An important aspect is that drug abuse potential, as well as, side effects are more dependent than time of exposition to the drug and the profile observed with the slow release formulation are compatible with this fact. On the other hand, reduction of weight due to loss of appetite seems not to depend on the C_{max} reached, but the time of exposure to the drug. Since both formulations reached similar through levels, it may be possible that reduction of body weight and therefore, body mass

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Table 2. Comparison of the pharmacokinetic parameters obtained after administration of clobenzorex immediate release and slow release formulations. Data are expressed as mean ± s.e.m. of thirty subjects per group.

Parameter	Slow release formulation (60 mg)	Immediate release formulation (30 mg)	p-value
C_{max} (ng/ml)	1.777 ± 0.514	3.429 ± 1.187	<0.01
T_{max} (h)	6.183 ± 0.764	1.458 ± 0.187	<0.001
AUC (ng·h/ml)	17.770 ± 4.529	11.076 ± 3.917	<0.05
$T_{1/2}$ (h)	15.002 ± 4.946	3.118 ± 0.271	<0.001

Table 3. Through plasma levels reached after administration of 30 mg immediate release capsule of clobenzorex b.i.d. and 60 mg slow release tablet once a day. Data are expressed as mean ± s.e.m. of 30 subjects per group. No statistically significant difference between treatments was observed.

Day of treatment	Concentration observed (ng/ml) after slow release formulation (60 mg)	Concentration observed (ng/ml) after immediate release formulation (30 mg)
7	1.0714 ± 0.1962	1.0897 ± 0.1799
14	0.9282 ± 0.1361	1.2280 ± 0.2494
21	0.9363 ± 0.1236	1.3314 ± 0.2357
28	1.3075 ± 0.3283	1.4925 ± 0.4671

Table 4. Cummulative body mass index and weight reduction during the treatment with clobenzorex 60 mg slow release tablet administered once a day and 30 mg immediate release capsule administered b.i.d. Data are expressed as mean ± s.e.m. No statistically significant difference between treatments was observed.

Day of treatment	Body weight reduction (kg)		BMI reduction (kg/m^2)	
	Immediate release capsule	Slow release tablet	Immediate release capsule	Slow release tablet
7	1.75 ± 0.28	1.44 ± 0.21	0.67 ± 0.11	0.53 ± 0.08
14	1.96 ± 0.29	1.90 ± 0.26	0.74 ± 0.11	0.70 ± 0.10
21	2.33 ± 0.38	2.44 ± 0.33	0.90 ± 0.14	0.91 ± 0.13
28	2.65 ± 0.38	2.57 ± 0.36	1.03 ± 0.14	0.95 ± 0.14

index will be similar. This was the case, since rate of reduction of body weight and body mass index was similar with the evaluated formulations.

In conclusion, clobenzorex slow release tablet shows an adequate pharmacokinetic profile and therefore, it is a good alternative for supporting body weight reduction, additional to diet and exercise, since its anorexigenic activity with this formulation should be mantained with a once a day administration.

5. Acknowledgments

Authors kindly acknowlegde Productos Medix, S. A. de C. V., Mexico City, Mexico for the formulations gifted in this study.

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Letter to the Editor

Amphetamine-Positive Drug Screens from Use of Clobenzorex Hydrochlorate

To the Editor:

Clobenzorex hydrochlorate, supplied under the trade name Asenlix, is an anoretic drug that is being prescribed in Mexico. The Mexican Physicians' Desk Reference (1) describes this drug as especially useful in patients that have diabetes, cardiac impairments, hypertension, anxiety, hyperactivity, and psychiatric illnesses. They attribute this to a low degree of central nervous system activity and "physical excitation".

Our interest in this drug was prompted by multiple incidences of amphetamine-positive drug screens in which the client called back and reported that the employees were taking Asenlix that was prescribed for them by their doctors in Mexico. Looking at the structure, it is plausible that amphetamine is a metabolite of clobenzorex (Figure 1).

To prove this metabolic pathway, we obtained a capsule of Asenlix containing 30 mg of clobenzorex hydrochlorate. A drug-free volunteer took this as a single dose. Urine specimens were collected at 1, 3, 4.5, and 7 hours post-ingestion. These were analyzed for amphetamine by GC/MS after an initial screen by TDx. The specimens obtained at 3, 4.5, and 7 hours all screened greater than the cutoff (1 μg/mL) on the TDx. GC/MS results are shown in Table I.

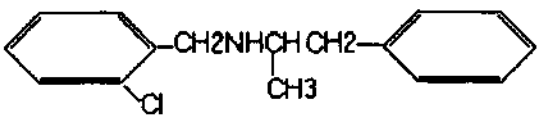

Figure 1. Structure of clobenzorex hydrochlorate.

Table I. GC/MS Results after 30-mg Dose of Clobenzorex Hydrochlorate

Time of specimen collection	Amphetamine concentration
1 h	51 ng/mL
3 h	840 ng/mL
4.5 h	1228 ng/mL
7 h	1365 ng/mL

Laboratories, especially those doing drug screens for clients near the border of Mexico or that have populations with frequent contact with Mexico, need to be aware that physicians there are prescribing Asenlix and that it will cause a positive drug screen for amphetamine.

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Reference

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Clobenzorex: Evidence for Amphetamine-Like Behavioral Actions

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YOUNG, R; N. A. DARMANI; E. L. ELDER; D. DUMAS AND R. A. GLENNON. *Clobenzorex: Evidence for amphetamine-like behavioral actions.* PHARMACOL BIOCHEM BEHAV **56**(2) 311–316, 1997.—Clobenzorex, an optically active *N*-substituted derivative of (+)amphetamine, has been identified on the illicit market. Because so little is known regarding the pharmacology or abuse potential of this agent, it was examined in tests of stimulus generalization in rats trained to discriminate 1 mg/kg of (+)amphetamine from vehicle to determine if it would produce amphetamine-appropriate responding. Clobenzorex (ED_{50} = 6.6 mg/kg) substituted for (+)amphetamine (ED_{50} = 0.3 mg/kg) but was approximately twenty times less potent than the training drug. Clobenzorex was also compared with (+)amphetamine and cocaine for its ability to induce locomotor stimulation and rearing frequency in mice. Clobenzorex was active in both assays but was less potent than either (+)amphetamine or cocaine. It is concluded that, although weaker than (+)amphetamine, clobenzorex constitutes an agent with amphetamine-like central stimulant behavioral properties. **Copyright © 1997 Elsevier Science Inc.**

(+)Amphetamine Clobenzorex Cocaine Drug discrimination Locomotor activity Rearing behavior Central stimulants Anorectics

CLOBENZOREX or S(+)1-phenyl-2-[(2-chorobenzyl)amino]propane (see Fig. 1 for structure), is an amine-substituted derivative of S(+)amphetamine available in Mexico, Spain, Argentina, and France as an anorectic agent (17). Amphetamine is a prototypical phenylalkylamine stimulant, and optimal stimulant potency is associated with a primary amine, or a secondary amine where the amine substituent is a methyl group (12). Amphetamine derivatives with amine substituents larger than a methyl group typically display reduced central stimulant properties (12,25). Indeed, it has been suggested that clobenzorex differs from amphetamine as an anorectic in that it doesn't produce central excitation or insomnia at therapeutic doses (19). The anorectic potency and toxicity (LD_{50} = 103 mg/kg; mouse) of clobenzorex is also less than that of (+)amphetamine (1). Although there have been reports describing the spectroscopic and structural character of clobenzorex (e.g., (2,3,10,18) extraordinarily little has been published on its pharmacology since it was first reported by Boissier et al in 1966 (1).

Clobenzorex recently came to our attention when it was identified as an adulterant or contaminant of some Chinese herbal medicines by the National Laboratory of Foods and Drugs in Taiwan (Dr. Y. Sheu, personal communication). There is almost no literature on clobenzorex as a potential drug of abuse. For example, clobenzorex appears to have been responsible for producing an acute psychosis, including visual and auditory hallucinations, in one individual, but this response may have resulted from a drug interaction (16). There is also a report of abuse by another individual who was reportedly addicted to clobenzorex (5). With little compelling evidence supporting the possibility that clobenzorex may be an amphetamine-like agent, even though clobenzorex has been encountered recently in forensic samples (18), we thought that further evaluation of this agent was in order.

Being a derivative of the central stimulant amphetamine, clobenzorex (if indeed a central stimulant) may behave in a manner similar to that of amphetamine. Amphetamine and amphetamine-like agents produce significant stimulant effects

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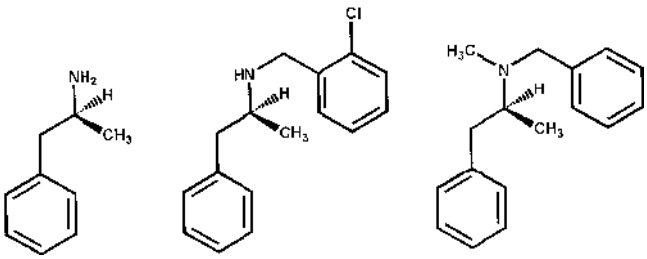

FIG. 1. Structures of S(+)amphetamine (left), clobenzorex (center), and S(+)*N*-benzphetamine (right).

in mice (e.g., (12,22); this can be observed by measuring such behaviors as locomotor stimulation and rearing. This is one of the oldest and most widely used methods for assessing the stimulant activity of drugs. Another standard technique for detecting amphetamine-like activity is to examine agents in tests of stimulus generalization in animals (e.g., rats, pigeons) trained to discriminate (+)amphetamine from vehicle (reviewed: 14,26). However, it has been demonstrated that the stimulus properties of amphetamine are not necessarily related to its stimulant actions; that is, the locomotor effects of stimulants may involve dopaminergic pathways in the striatum whereas the stimulus effects of amphetamine seem to be mediated by mesolimbic dopaminergic systems [see Goudie (14) for discussion]. Thus, use of two different types of measures, central stimulation in mice and stimulus generalization in rats trained to discriminate (+)amphetamine, not only offers two independent means of assessing the activity of clobenzorex, but also provides us with data in two species of animals. Furthermore, although many amphetamine-related anorectic agents (e.g., phenmetrazine, phendimetrazine, diethylpropion), as well as some non-amphetaminergic anorectics (mazindol), are capable of producing amphetamine-like stimulus effects, not all (e.g., fenfluramine, benzphetamine) anorectics reliably produce this effect (e.g., (8,26). Certain amphetamine-related anorectics (e.g., phenmetrazine, diethylpropion, phenteramine), as well as mazindol and amphetamine itself, also substitute for cocaine when cocaine is used as training drug, whereas others (e.g., fenfluramine) do not (24). Consequently, we examined doses of clobenzorex in tests of stimulus generalization in rats trained to discriminate 1 mg/kg of (+)amphetamine from vehicle, and for its ability to produce locomotor stimulation and rearing in mice relative to (+)amphetamine and cocaine. Significant effects in all assays would suggest that clobenzorex possesses central stimulant properties.

METHODS

Drug Discrimination Studies

Five naive male Sprague–Dawley rats (ca. 250–300 g), housed individually, were reduced in body weight to approximately 80% of their free-feeding weight. During the entire course of the study, the animals' body weights were maintained at this level by partial food deprivation; in their home cages, the animals were allowed drinking water ad lib. The animals were trained (15-min training session) to discriminate intraperitoneal injections (15-min presession injection interval) of 1.0 mg/kg of (+)amphetamine sulfate from vehicle (sterile 0.9% saline) under a variable interval 15-sec schedule of reinforcement for appetitive (sweetened powdered milk) reward. Standard two-lever operant chambers (Coulbourn Instruments model E10-10) were used. In general, daily training sessions were conducted with (+)amphetamine or 1.0 ml/kg of saline; on every fifth day, learning was assessed during an initial 2.5-min non-reinforced (extinction) session followed by a 12.5-min training session. For approximately half the animals, the left lever was designated the drug-appropriate lever whereas the situation was reversed for the remaining animals. Data collected during the extinction session included responses per minute (i.e., response rate) and number of responses on the drug-appropriate lever (expressed as a percent of total responses). Animals were not used in stimulus generalization studies until they made $> 80\%$ of their responses on the drug-appropriate lever after administration of training drug, and $< 20\%$ of their responses on the same drug-appropriate lever after administration of saline, for three consecutive weeks. The animals were placed in the operant chambers no more than once per day and were in their home cages except during training and extinction sessions.

Tests of stimulus generalization were conducted in order to determine if clobenzorex would substitute for the (+)amphetamine stimulus. During this phase of the study, maintenance of the training drug discrimination was insured by continuation of the training sessions on a daily basis (except on a generalization test day; see below). On one of the two days before a generalization test, approximately half of the animals would receive training drug and half would receive saline; after a 2.5-min extinction session, training was continued for 12.5 min. Animals not meeting the original criteria (i.e., $>$ 80% of total responses on the drug-appropriate lever after administration of training drug and $< 20\%$ of total responses on the same lever after administration of saline) during the extinction session were excluded from the immediately subsequent generalization test session. During the investigations of stimulus generalization, test sessions were interposed among the training sessions. The animals were allowed 2.5 min to respond under non-reinforcement conditions; the animals were then removed from the operant chambers and returned to their home cages. An odd number of training sessions (five) separated any two generalization test sessions. Doses of the challenge drugs were administered in a random order, using a 15-min presession injection interval. Stimulus generalization was said to have occurred when the animals, after a given dose of challenge drug, made r 80% of their responses on the drug-appropriate lever. Animals making fewer than 5 total responses during the 2.5-min extinction session were considered as being disrupted. ED_{50} values (i.e., doses where the animals would be expected to make 50% of their responses on the drug ~appropriate lever) were calculated by the method of Finney (9). Solutions of all drugs were prepared fresh daily using 0.9% sterile saline. All drugs were administered via intraperitoneal injection 15 min prior testing. A total of four doses of (+)amphetamine (0.1, 0.25, 0.5, and 1.0 mg/kg) and six doses of clobenzorex (1.5, 3.0, 7.5, 9.0, 12.0 and 14.0 mg/kg) were examined.

Locomotor Activity and Rearing

Albino ICR mice (male and female) weighing 25–30 g were used throughout the study. Male and female mice were housed separately in groups of five on a 12-h L: 12-h D cycle at a room temperature of 21 $\pm$ 1°C with free access to food and water. All experiments were performed between 0800 and 1700 h. On the test day, animals were acclimated in black plastic dummy observation cages ($28 \times 28 \times 28$ cm) for 30 min prior to drug administration. The animals were then injected

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intraperitoneally with either vehicle (water, n = 6–7) or varying doses of either amphetamine (0.1, 0.5, 1.0, 2.0 mg/kg, n = 6–7/group), cocaine (5.0, 10.0, 20.0 mg/kg, n = 6–7/group) or clobenzorex (5.0, 10.0, 20.0 mg/kg, n = 6–7/group). Following these injections, the animals were returned to the dummy observation cages. Ten minutes later, the mice were transferred and placed individually in four black experimental observation cages of similar dimension. Both locomotor activity and rearing frequency were recorded simultaneously for the next 40 min by a computerized video tracking, motion analysis, and behavior recognition system [Ethovision (version 1.7), Noldus Information Technology, Costerweg, Netherlands]. In order to see whether clobenzorex might have a long duration of action, the morning-treated animals were again observed, for 40 min as described above, 3 h after the initial injection. The data were analyzed either for the total 40-min session or at 10-min intervals for the 40-min observation period. Different versions of this system have been previously used for determination of locomotor activity (i.e., distance traversed) in different animal species (20,23). Version 1.7 of this system also automatically detects rearing frequency. Rearing behavior in rats has been validated (Ethovision manual, version 1.7). The detection of rearing is based on the change in the surface area of the animal as seen by overhead video camera when the animal stands on its hind legs. Our preliminary experiments indicated that a 5% change in surface area for mice is equivalent to 90% to 110% of manual recording of rearing frequency. Thus, a 5% setting in surface area change was used throughout the experiments.

Drugs

(+)Amphetamine sulfate and cocaine hydrochloride were purchased from Sigma Chemical Company. Clobenzorex was synthesized in our laboratory from (+)amphetamine and isolated as its hydrochloride salt. A mixture of equimolar (13.4 mmol) amounts of (+)amphetamine (free base), 2-chlorobenzyl chloride, and potassium carbonate in xylene was heated under reflux for 3 h; water was added and the mixture was extracted with xylene (3 × 10 ml). The organic portion was washed successively with water (2 × 10 ml) and brine (10 ml), dried ($MgSO_4$), and evaporated to dryness. The crude product was purified by column chromatography (silica gel, EtOAc/petroleum ether 2:8) to give the free base of clobenzorex as a pale yellow oil which was homogeneous by thin layer chromatography. An ethereal solution of the free base was treated with HCl gas at 0°C to obtain the salt as white crystals. Infrared (film, free base) and proton magnetic resonance (GE Q-300; HCl salt in deuterated water) spectra were consistent with the assigned structure. The melting point (176–178°C) and optical rotation ($[\alpha]_D^{20}$ = +25.5 ± 1.0; 1%, H_20) of the salt were consistent with what has been previously published in the literature (mp = 182–183°C; $[\alpha]_D^{20}$ = +26.3 ± 1.0) (1); (mp = 174–176°C) (18).

Statistics

Data were analyzed by one way analysis of variance (ANOVA), and post hoc analysis was performed by Dunnetts t-test or Fishers PLSD test. For comparison of morning versus afternoon data (locomotor and rearing experiments), a 2-factor repeated measure of ANOVA was used. A p value of < 0.05 was necessary to achieve statistical significance.

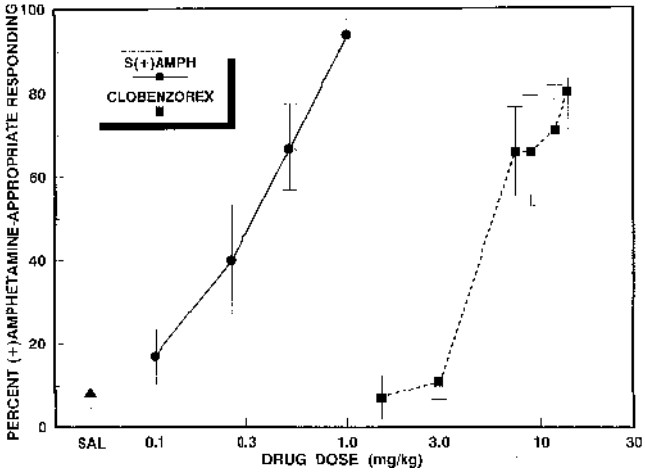

FIG. 2. Percent (mean ± SEM.) amphetamine-appropriate responding occasioned by 1 ml/kg of saline (SAL), and doses of (+)amphetamine (circles) and clobenzorex (squares) 15 min following administration to rats (n = 5) trained to discriminate 1 mg/kg of (+)amphetamine from saline vehicle.

RESULTS AND DISCUSSION

Drug Discrimination Studies

The results of the drug discrimination studies demonstrate that clobenzorex is capable of producing amphetamine-like effects; Figure 2 shows that the (+)amphetamine stimulus (ED_{50} = 0.3 mg/kg; 95%CL = 0.1–0.6 mg/kg) generalizes to clobenzorex (ED_{50} =6.6 mg/kg; 95%CL = 4.0–10.8 mg/kg). The animals' response rates following the administration of doses of clobenzorex (10.6–15.3 responses/min) were similar to those following administration of doses of (+)amphetamine (11.9–14.7 responses/min) or saline (14.5 responses/min). All five animals responded under all conditions except that one animal was disrupted following administration of 14 mg/kg of clobenzorex. In the drug discrimination assay then, clobenzorex (ED_{50} = 25.3 μmoles/kg) is about twenty times less potent than (+)amphetamine (ED_{50} = 1.2 μmoles/kg).

Central Stimulation in the Mouse

Administration of low to moderate doses of clobenzorex (i.e., 5 and 10 mg/kg) to groups of 6-7 mice showed no significant increase in locomotor activity over water control. At a dose of 20 mg/kg, locomotor stimulation was significantly greater than that for control [$F(4, 29)$ = 41.4, $p < 0.0001$] (Fig. 3), and was approximately half that obtained with 2 mg/kg of (+)amphetamine or 20 mg/kg of cocaine. The 20 mg/kg clobenzorex-treated animals were also examined three h post injection (data not shown); at this time, locomotor activity was not significantly different from those animals that had been treated three h earlier with water. The effect of clobenzorex on rearing frequency (Fig. 4) essentially paralleled the effect on locomotor activity. That is, 5 and 10 mg/kg of clobenzorex produced no significant increase in rearing frequency; 20 mg/kg of clobenzorex increased rearing frequency by about 40% over control. At 2 mg/kg of (+)amphetamine and 20 mg/kg of cocaine, rearing frequency increased by about 60% and 70%, respectively, over water control. At 20 mg/kg, clobenzorex produced about 75% of the increase in rearing frequency obtained with 20 mg/kg of cocaine.

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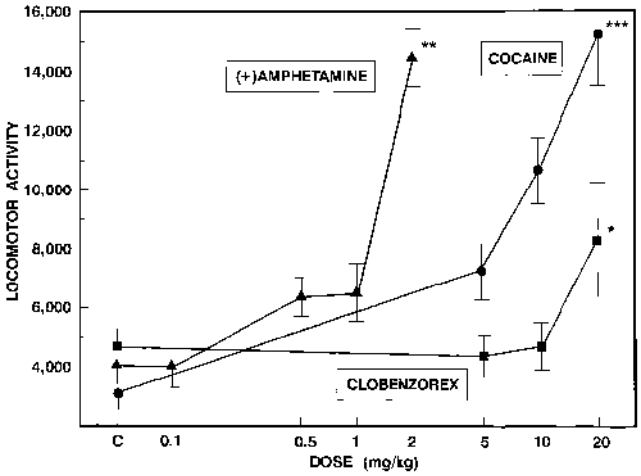

FIG. 3. Effect (mean ± SEM.) of (+)amphetamine, cocaine, and clobenzorex doses on the locomotor activity of mice ($n = 6$–7 animals/dose). Locomotor activity is expressed as the distance traversed (in cm) per 40 min; C = the effect of water administration. * = Significantly different from control by Dunnett t-test [$F(7, 46) = 4.1$, $p < 0.001$]. ** = Significantly different from control by Dunnett t-test [$F(4, 29) = 41.4$, $p < 0.0001$]. *** Significantly different from control by Dunnett t-test [$F(3, 23) = 27.7$ $p < 0.0001$].

Locomotor activity following administration of 20 mg/kg of clobenzorex was monitored for 40 min in 10-min intervals. Actual distance traversed (in cm) for each of the 10-min periods (following the initial 10-min delay) is as follows: 0-10 min (2745 ± 507), 10-20 (1980 ± 545), 20–30 min (1795 ± 526), and 30-40 min (1760 ± 298). That is, there was no progressive increase in activity per successive 10-min period ($p > 0.05$).

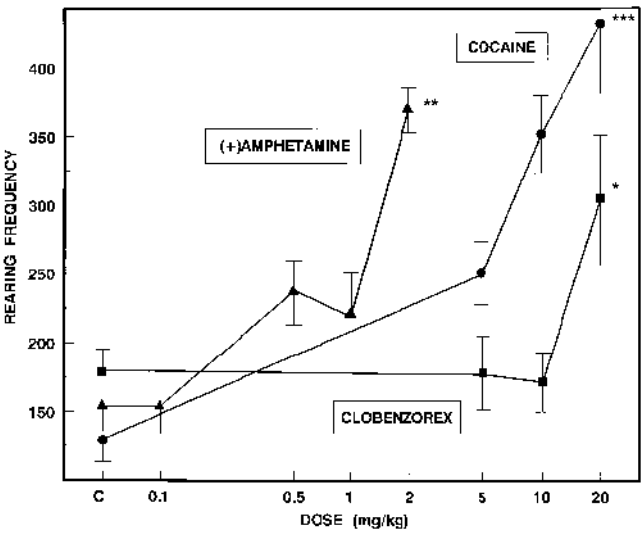

FIG. 4. Effect (mean ± SEM.) of (+)amphetamine, cocaine, and clobenzorex doses on the frequency of rearing using mice ($n = 6$–7 animals/dose) as subjects. Rearing frequency is expressed as the number of rearings occurring in a 40-min period; C = the effect of water administration. * = Significantly different from control [$F(7, 46) = 2.89$, $p < 0.01$]. ** = Significantly different from control [$F(4, 29) = 17.1$, $p < 0.0001$]. *** = Significantly different from control [$F(3, 23) = 19$, $p < 0.001$].

Similar results were obtained with rearing frequency for the corresponding four time periods (i.e., 85 ± 12, 66 ± 15, 62 ± 15, and 69 ± 10 rearings).

Clobenzorex as an Amphetaminergic Agent

(+)Amphetamine is a central stimulant that serves as a discriminative stimulus in animals (26). *N*-Monomethylation of amphetamine results in enhancement of potency both in studies of locomotor stimulation (22) and in tests of stimulus generalization (26). Substitution on the terminal amine of amphetamine with progressively larger alkyl groups results in a progressive reduction in potency (12,13,22,25). For example, *N*-benzylamphetamine is only 8% as potent as amphetamine as a central stimulant in mice (22); benzphetamine (Fig. 1), the *N*-benzyl analog of methamphetamine, is also a weaker central stimulant than amphetamine (6). In tests of stimulus generalization using pigeons, monkeys, or humans trained to discriminate amphetamine from vehicle (or placebo), results are mixed. In pigeons, stimulus generalization failed to occur up to doses of 100 mg/kg (8). Although benzphetamine substituted for (+)amphetamine in three of four monkeys (7), a majority of human subjects failed to recognize doses of up to 50 mg of benzphetamine as being like amphetamine (4).

Clobenzorex, *N*-benzylamphetamine, and benzphetamine all share a common *N*-benzyl substituent; in clobenzorex, the *N*-benzyl group further possesses an ortho chloro substituent. As such, given the established structure-activity relationships described above, it might be expected that because clobenzorex bears a rather large *N*-substituent it would be, at best, a weak amphetamine-like agent. This expectation is supported by the results of the present investigation. In tests of stimulus generalization using amphetamine-trained rats clobenzorex substitutes for (+)amphetamine but is approximately 20-fold less potent than the training drug. Likewise, clobenzorex behaves as a central stimulant in mice as determined by its effects on locomotor activity (Fig. 3) and rearing (Fig. 4). The results of both of the latter assays suggest that clobenzorex possesses amphetamine-like stimulant character. Although it is rather difficult to make quantitative comparisons with these latter data, the results are not inconsistent with the results of the drug discrimination studies which suggest that clobenzorex is approximately twenty time less potent than (+)amphetamine. It is concluded, on the basis of drug discrimination studies using rats trained to discriminate (+)amphetamine from saline vehicle, as well as on tests of locomotor activity and rearing frequency in mice, that clobenzorex possesses amphetamine-like behavioral character, but is less potent than (+)amphetamine itself.

Possible Role of Metabolism

One final question that should be considered is whether clobenzorex is active per se or whether it is first metabolized to an active metabolite. *N*-benzylamphetamine derivatives are known to undergo *N*-debenzylation in rodents and in humans (e.g., see (15), but the *N*-debenzyl derivatives do not appear to be major metabolites (15). Benzphetamine, for example, is metabolized in rodents and humans, to amphetamine and methamphetamine, but these constitute minor metabolites (15). However, the added role that the chloro substituent of clobenzorex might have on debenzylation is unknown. Thus, the possibility exists that clobenzorex might be metabolized to amphetamine. Indeed, a search of the literature reveals that a single investigation of clobenzorex metabolism has been

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reported (11). In rats, 24 h after intraperitoneal clobenzorex administration, amphetamine, *para*-hydroxyamphetamine, and *para*-hydroxyamphetamine conjugates account for 5.5%, 3.1% and 12.7%, respectively, of the administered dose; the major urinary metabolite (40%) is clobenzorex glucuronide (11). Amphetamine has also been identified as a metabolite of clobenzorex in one human volunteer (21). Although clobenzorex can be metabolized to amphetamine, and although clobenzorex is rapidly absorbed and reaches a maximal blood level in rodents 5 min post administration (11), the extent to which clobenzorex is metabolized to amphetamine within 15–50 min (i.e., times used in the present investigation) is unknown.

Approximately 20% of a clobenzorex dose can be accounted for (after 24 h) as amphetamine or an amphetamine metabolite (11); if metabolism occurs very quickly, this might account for the potency of clobenzorex being approximately 20% that of (+)amphetamine in the present investigation. This is particularly possible in the locomotor and rearing studies where activity was monitored until 50 min post injection (i.e., for 40 min following a 10-min delay) as opposed to the drug discrimination studies (where the effect of clobenzorex was examined for only 2.5 min, 15 min after drug administration). We explored this possibility by (a) examining the effect of 20 mg/kg of clobenzorex in successive 10-min blocks following administration to mice so as to note any progressive effect, and by (b) measuring locomotor activity three h post injection of 20 mg/kg of clobenzorex. In both the locomotor and rearing assays, the effect of clobenzorex was no greater at 10–20 min, 20–30 min, or 30–40 min than it was at 0–10 min. In the locomotor assay, the effect of 20 mg/kg of clobenzorex 3 h post administration was not statistically different than the effect seen three h following the administration of water. That is, there was no evidence to suggest that clobenzorex is any more effective after 40 min than it is after the first 10 min.

CONCLUSIONS

Clobenzorex was shown to possess amphetamine-like character both in rats and mice. Amphetamine-stimulus generalization occurs to clobenzorex, and clobenzorex is approximately 20 times less potent than (+)amphetamine. In this respect, it behaves like some other amphetamine-based anorectic agents (8). Clobenzorex is also less potent than (+)amphetamine and cocaine in producing locomotor stimulation and rearing in mice. Although there is evidence in the literature that clobenzorex is metabolized to amphetamine in rodents, it is unlikely that this can account for the stimulant properties of clobenzorex unless extensive metabolism occurs within the first 10 min after administration. This is consistent with previous reports on *N*-benzylamphetamine (i.e., deschloro-clobenzorex) which, although known to be metabolized to amphetamine (15), is only 8% as effective as amphetamine as a locomotor stimulant (22). Further work will be necessary to study the rate of metabolism of clobenzorex and the contribution (if any) of its metabolites to its actions.

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ACTA PSYCHIATRICA SCANDINAVICA

Case report

Amphetamine-like stimulant cessation in an abusing patient treated with bupropion

Tardieu S, Poirier Y, Micallef J, Blin O. Amphetamine-like stimulant cessation in an abusing patient treated with bupropion.
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Objective: Bupropion sustained release is considered to be a weak inhibitor of dopamine and serotonin reuptake.
Method: We report the case of an amphetamine-abusing patient who self-administered bupropion.
Results: Since 30 years, a 52-year-old women used amphetamine derivates. She explained her need for amphetamine use in order to perform daily activities. Recently, she decided to experiment with bupropion. She abruptly stopped taking clobenzorex and simultaneously started taking bupropion (150 mg/day). The seventh day she reported a concomitant intake of clobenzorex; this induced adverse effects. Whilst taking bupropion, she described experiencing an euthymic state without any compulsion to take amphetamine drugs and was able to perform daily activities. After stopping it, no symptoms of withdrawal were reported by the patient.
Conclusion: This observation supports an another report suggesting that bupropion may be of help in weaning from amphetamine users and should be confirmed by clinical trials.

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Key words: bupropion; substance; withdrawal syndrom; amphetamine; case report; human

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Introduction

Bupropion hydrochloride has structural similarities to catecholamines and amphetamine and is considered to be a weak inhibitor of dopamine and serotonin reuptake (1).

Bupropion sustained release (SR) is indicated as an aid to smoking cessation. Either alone or in combination with the nicotine patch, bupropion SR results in significantly higher short- and long-term smoking cessation rates than treatment with the nicotine patch alone or placebo (2, 3). Because of the similar discriminative stimulus effects between bupropion and cocaine, bupropion has been evaluated in cocaine dependence but led to disappointing results. Bupropion is not considered as an useful treatment agent for cocaine abuse (4). Clobenzorex [S-(+)-N-(2-chlorobenzyl)-amphetamine] is a N-substituted derivative of (+) amphetamine which possesses amphetamine-like central stimulant behavioral properties (5).

Case report

A 52-year-old woman with a 30-year history of amphetamine abuse was seen in hospital consultation.

She began to experience amphetamine (dexamphetamine tartrate) and other illicit product during University studies. No history of alcohol, tobacco, heroin, hashish was found. However several amphetamine derivates in particular clobenzorex, fenproporex, amfepramone were used by the patient with a liking preference for clobenzorex. The patient explained the need of amphetamine use in order to perform the daily activities and not for anorexic effects and weight control. Many times, she had tried to stop it without success. Since 1 year, she took fluoxetine (40 mg/day) for depressive state.

Recently, she decided to get bupropion seeing its similar amphetamine-like adverse effects in a medical review in a general practitioner. Brutally, she stopped clobenzorex at the beginning of the first intake of bupropion SR (150 mg once daily).

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The first intake was associated with a capsule of fluoxetine. This drug association was not well tolerated (palpitations, visual troubles). From the second day, she only took a single dose of bupropion SR (150 mg). Punctually (the seventh day) she reported a concomitant intake of two tablets of clobenzorex that induced several adverse effects (palpitations, irritability, anorexia, hyperactivity). During the bupropion SR intake, she described an euthymic state without any compulsion to take amphetamine drugs and was able to perform daily activities. No increase in irritability, restlessness, depression and sleep disorders or other adverse events were noted during bupropion treatment. Since the stop of bupropion (the fourtieth day), no symptoms of withdrawal and no needs to take any amphetamine derivate were reported by the patient.

Discussion

Given the importance of craving, withdrawal symptoms and maintenance treatment in the drug cessation process, the effects of bupropion on these parameters should be discussed respectively. There is today no evidence that bupropion diminishes drug craving. Indeed, no effect of bupropion has been observed on nicotine craving in humans (6). On the contrary, although bupropion demonstrates potency as reinforcer in animals, it has not been reported to induce addiction (7).

In comparison with placebo, short-term treatment of fluoxetine (40 mg/day) significantly decreases craving (8) and although our patient did stop fluoxetine at the beginning of bupropion treatment, a residual effect related to fluoxetine long half-life cannot be ruled out.

One alternative explanation would be that bupropion acted as a maintenance therapy, mimicking the effects of stimulant amphetamine. It has been previously reported that bupropion at doses up to 200 mg produces none of the self reported effects characteristics of the stimulant amphetamine in normal humans (9, 10), and that D-amphetamine does not generalize fully to bupropion at doses up to 400 mg in D-amphetamine trained subjects without drug histories (11). However, bupropion at doses up to 400 mg produces mild amphetamine-like self reported effects in experienced drug users (12), as was our patient. Finally, D-amphetamine and bupropion similarly increase several stimulant-like properties (11). An alternative explanation is that bupropion would have facilitated amphetamine abstinence. In our case, the time course of the amphetamine cessation with bupropion was consistent with the previously reported effects on smoking cessation (2). Bupropion does not increase ratings of drug liking (11) and in the subgroup of depressed methadone-maintained cocaine abusers, bupropion has been suggested to facilitate cocaine abstinence (4). However, it is unlikely that the observed effects were related to antidepressant effects of either bupropion or fluoxetine taken by our patient. It is known that bupropion is efficacious for smoking cessation independently of a former history of major depression (13). Moreover, fluoxetine and venlafaxine cannot aid smoking cessation (14). Although fluoxetine has been reported to enhance retention in a naltrexone treatment program (15), fluoxetine has not been found to avoid relapse of cocaine abusers who were abstinent at the start of treatment (16).

In our case, increased self-reported cardiovascular effects were observed after concomitant intake of clobenzorex and bupropion. This observation differs from Oliveto results showing that bupropion does not alter the acute subjective or cardiovascular effects of cocaine (17). Therefore, an antabuse effect may also have play here a role.

An effect of bupropion on withdrawal symptoms diminution has also been suggested, both at low doses in pentobarbital-dependent rats (18) and in humans on some nicotine withdrawal symptoms (6). However, in marijuana withdrawal, bupropion has been reported to increase the withdrawal symptoms (19). In our case, no withdrawal symptoms has been reported, possibly because of the inhibition of dopamine reuptake which is a common pharmacological property of both bupropion and clobenzorex.

This observation supports another report (20) suggesting that bupropion may be of help in weaning from amphetamine users and should be confirmed by clinical trials.

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Invited comment

Tardieu et al. reported a fascinating case of a 52-year-old woman with a history of 30 years of amphetamine abuse. Self administration of bupropion was accompanied by a rapid and successful cessation, an outcome desired by herself. The case description represents a colorful illustration of the phenomenological and possible background mechanisms of amphetamine addiction (S. Tardieu, Y. Poirier, J. Micallef & O. Blin Unpublished data).

Amphetamines produce their effects on behavior by potentiating monoaminergic transmission at the cellular level. They increase dopamine (DA), serotonin (5-HT) and norepinephrine (NE) neurotransmission by acting as a substrate for their reuptake transporters, causing transmitter release from the presynaptic terminal into the synapse (1). Among these, the dopamine reuptake transporter (DAT) is most directly related to the reinforcing effects of psychostimulants (2). Amphetamines have the ability to activate mesocorticolimbic DA projections as substrates of a system rewarding and reinforcing effects of natural stimuli. These projections originate in the ventral tegmental area (VTA) and project to parts of the ventral striatum [Nucleus Accumbens (NAc)] and the prefrontal cortex (3). Considering the cellular activation level of the reward system, the reported case of a long term abusing woman probably represents a state of neuroadaptation because of chronic amphetamine ingestion. If drugs are repeatedly administered and then withdrawn, there is a significant increase in the threshold for achieving reward. The brain reward pathways could be in a state of decreased responsiveness, as when a homeostatic cascade would yield compensatory adaptations to excessive DA stimulation (4). For example, within the cellular cascade of signal transduction from the surface to the core, the up-regulation of the neuropeptide dynorphin could be one mechanism that could contribute to aversive states associated with psychostimulant withdrawal. In the NAc, levels of prodynorphin mRNA and dynorphin peptides increased significantly following repeated administration of psychostimulants (5). Dynorphin peptides are stimulating selectively the κ opiate receptors on DA synaptic terminals, followed by a decrease in DA release. The activation of κ receptors is associated behaviorally with an aversive dysphoric syndrome (6). Thus, in terms of controlling negative-feedback mechanisms, increased dynorphin produced by chronic amphetamine administration could inhibit DA release and contribute to emotional aspects of amphetamine withdrawal. The regulation of prodynorphin gene expression by psychostimulants again is dependent on DA stimulation. This involves in a more complex way parts of the intracellular cAMP-pathway, including CREB, a transcription factor that binds cAMP response elements (CREs), locking the circle of negative feedback (7).

Taking the above outlined neurobiological background into account, the issue of self administration of bupropion could have been instrumental in the case of cessation of long lasting amphetamine abuse. Bupropion is the only antidepressant that

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more potently blocks the reuptake of DA than that of either NE or 5-HT. However, bupropion is a weak inhibitor of monoamines (8). Among the explanations reasonably mentioned by Tardieu et al., it is imaginable that bupropion could have acted as maintenance therapy, even when it was administered at a low dose. Of course, bupropion could have facilitated amphetamine abstinence as well (9). However, are there other than neuropharmacological explanations possible, explaining rapid and successful cessation of long term psychostimulant abuse?

Unfortunately, we don't have a functional analysis of the addictive behavior of the 52-year-old woman. We do not know much about her assumptions, automatic thoughts and environmental influences. It could have been hypothesized that this patient has initiated a kind of 'placebo-reward' by repeating a self medication pattern, which began 30 years ago (10). In terms of associative learning and classic conditioning, a kind of generalization and semantic conditioning could have taken place. In other words, within a process of interoceptive conditioning, bupropion got the same meaning as amphetamine to the patient, when she was reading the medical information of the antidepressant at the general practitioner. As a kind of inhibitory contra conditioning, the hypothesized 'bupropion-placebo-reward' could have antagonized anticipated anhedonic and withdrawal symptoms, thus raising the odds for a successful cessation. That psychological and environmental aspects are of great importance in addictive behavior is known for decades, and was demonstrated by estimating relapse frequency of addicted Vietnam-veterans compared with civil addicts in the US. Approximately 80% of the soldiers were addicted to heroin. However, only a minority continued their addictive behavior after return home, despite the continued availability of drugs. In other words, external incentives and conditioned stimuli of the war situation were not available anymore in the US (11). Furthermore, relapse often occurs on re-exposure to cues associated with drug use, even years after any withdrawal symptoms have subsided. This is consistent with an important role for associative learning (12).

Neuropharmacological and cognitive psychological approaches in understanding and treatment of addiction do not need to conflict with one another. Associative learning is thought to represent long lived alterations in patterns of synaptic connectivity that encode specific memory information (13). Recent neuroimaging evidence expands the traditional concepts of drug dependence which are emphasizing limbic-regulated responses to pleasure and reward. The orbitofrontal cortex is involved in amphetamine addiction as well (14). Thus, neuroimaging studies as an example of neurobiological approaches could build a bridge to cognitive psychological work in this field. Further psychological as well as neurobiological research is needed to examine the therapeutical effects of pharmacological and psychological interventions in amphetamine addiction. From a clinician's point of view, both treatment parts belong to the desired standard of therapy.

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Orlistat

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Abstract

- **Treatments for obesity are disappointing. None has yet shown an effect on morbidity and mortality. Nondrug treatments are poorly assessed. Stable long-term weight loss necessitates long-term management.**
- **Orlistat (Xenical®, Hoffman–La Roche), a gastrointestinal lipase inhibitor, is indicated, in combination with a low-calorie diet, for management of obesity.**
- **The assessment file is bulky and methodologically sound, at least in terms of the weight loss end point.**
- **During medium-term trials (12 to 24 months), orlistat administered at a dose of 120 mg three times daily and combined with dietary intervention had a moderate positive effect on body weight (-3.5 kg on average).**
- **No longer-term trials have been done.**
- **It is unknown whether this drug affects morbidity and mortality linked to obesity.**
- **In clinical trials, patients treated with orlistat had an increased frequency of breast cancer. This potential risk is currently being assessed in a specific trial.**
- **Gastrointestinal adverse effects are frequent.**
- **Treatment is costly.**

JUDGMENT RESERVED

It is currently unknown whether orlistat, in addition to its moderate effect on weight loss, has any tangible benefits in terms of morbidity or mortality for obese people. There is also doubt over the risk of breast cancer associated with orlistat; the risk-benefit ratio cannot yet be established.

Orlistat

Capsules

- **120 mg of orlistat per capsule**

Cost for one capsule three times daily for 1 month is between $120 and $150 (without dispensing fee). Information is supplied by David Freeman, Pharmacist at the Orillia Soldiers Memorial Hospital in Ontario.

Licensed indication

Orlistat is indicated, in conjunction with a mildly hypocaloric diet, for treating obese patients (body mass index [BMI] ≥30 kg/m^2) or overweight patients (BMI 28 kg/m^2) with associated risk factors. Orlistat must not be used by children.

Gastrointestinal lipase inhibitor; antiobesity drug

Obesity is a public health problem affecting 6% to 10% of adults.[1] According to epidemiologic studies, mostly done in the United States, obesity (BMI ≥30 kg/m^2 [a]) correlates with higher mortality, most excess deaths being due to cardiovascular events.[2]

Obesity not only disturbs daily life activities, but also increases morbidity, especially from cardiovascular causes. It requires long-term management (perhaps throughout life) to stabilize body weight after an initial phase of weight loss.[2]

Current treatments for obesity are disappointing (see sidebar). More than 80% of people who lose weight gradually regain it.[2] No treatments for obesity have demonstrated positive effects on cardiovascular mortality or morbidity. It has even been suggested that repeated body weight fluctuations might increase mortality.[2]

The main end point of treatment for obesity should be reduced morbidity and mortality. Weight loss itself should be only a secondary end point.

Several drugs have been touted as adjuvant treatments for obesity. Some, such as dexfenfluramine (Redux), have been withdrawn from the market because of their adverse effects.[3] Other appetite suppressants have been kept on the market, but cannot be used for long periods because of the risks of addiction and pulmonary hypertension (b).[4] Other drugs (eg, bulk-forming agents, anti-edema drugs and lipolytic

a—*Body mass index is calculated as weight divided by height squared (w/h^2): weight in kilograms, height in metres.*[2]

b—*The amphetamine appetite suppressants currently marketed for this indication are amfepramone, clobenzorex, and fenproporex.*

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agents, phytotherapy) have no proven specific clinical activity.

In a context of high patient and prescriber expectations, we examine whether the market release of orlistat (Xenical®, Hoffman–La Roche) represents an advance in management of obesity, and an increase in the benefits that can be expected in terms of mortality, morbidity, and weight loss.

Assessment limited to secondary end points

Orlistat is a lipase inhibitor. Administered orally, it is poorly absorbed and acts by partially inhibiting gastrointestinal lipase activity.[5] At recommended doses, and when the diet contains approximately 30% of calories in the form of lipids, orlistat leads to daily stool excretion of about 20 g of undigested unabsorbed fat, an amount equivalent to 180 kcal.[6]

The clinical file on orlistat is methodologically sound. Reports of assessment of weight loss make the file bulky, as do those of evaluation of cardiovascular risk factors. In contrast, no trials conducted to date have end points based on obesity-related morbidity or mortality.

Optimal dose: 120 mg at each of three meals? Four randomized trials were conducted to determine the optimal dose of orlistat. They lasted 2 to 6 months and involved 1421 patients.[7] The largest study, and the only one to assess a dose of 240 mg three times daily (tid), involved 676 subjects with a BMI between 28 and 43 kg/m^2.[8] Four doses of orlistat (30 mg, 60 mg, 120 mg, and 240 mg) administered tid were compared with placebo. All subjects also followed a diet that aimed to create an energy deficit of 500 kcal/d (low-calorie diet).

The treatment period was 24 weeks. Intention-to-treat analysis was carried out. The main end point was weight loss. Weight loss was significantly higher among those receiving orlistat at doses of 60 mg, 120 mg, and 240 mg tid than among those receiving placebo. The group receiving 120 mg tid showed the biggest weight loss.[8] In trials that lasted a year, however, 120 mg tid was no more effective than 60 mg tid.

Seven medium-term placebo-controlled trials. Seven randomized double-blind trials (four American and three European multicentre studies) lasting between 1 and 2 years involved 4188 patients older than 18, with a BMI between 28 and 47 kg/m^2 (sex distribution not given).[7] Only three of these trials have been published in detail,[9-11] but we had access to a meta-analysis of the data in the European Medicines Evaluation Agency report.[7]

All the trials were placebo-controlled. None compared orlistat with another treatment for obesity.

Weight loss was always the main end point. The other end points were changes in cardiovascular risk factors (serum glucose levels, plasma lipids, blood pressure).

Six of the seven trials comprised a preliminary low-calorie diet phase with placebo lasting 4 to 5 weeks to select highly motivated, adherent patients. Randomization took place after this initial phase.

Four of the trials lasted 2 years and had two distinct phases: the effect of orlistat combined with a low-calorie diet (below 1500 kcal/d, including 30% lipids) was assessed during the first year (weight-loss phase), and the effect of orlistat combined with a diet aimed at stabilizing weight loss (two trials) (**c**) or reducing weight resumption (two trials) (**d**) was assessed during the second year. Results achieved in the weight-loss phase of these four trials (and a 1-year trial with an identical design) formed the basis for a meta-analysis.[7]

Another trial lasting 1 year assessed the effect of orlistat plus a low-calorie diet on patients with type 2 diabetes who were overweight or obese.[10]

The seventh trial assessed the effect of 1 year of treatment with a low-calorie diet and orlistat on body weight stabilization. Initial weight loss was achieved by means of a 6-month low-calorie diet alone.

Weight loss during the first year of treatment in some subjects only. In the meta-analysis of the five placebo-controlled trials testing the effect of orlistat plus a low-calorie diet, mean weight loss between randomization and the end of the 12th month of treatment was 6.1 kg in the groups receiving 120 mg orlistat tid and 2.6 kg in the placebo groups ($P<.001$; mean weight at randomization, 97 kg).[7] The proportion of patients who lost 10% or more of their initial weight was higher

c—*The stabilizing diet was prescribed as follows: patients who lost less than 3 kg in the last 2 months of the first year did not modify their diet, while patients who lost 3 kg or more during the last 2 months of the first year increased their daily calorie intake by 200 to 300 kcal.*

d—*To evaluate the effect of orlistat on maintenance of weight loss, the two trials had a second randomization at the end of 1 year of treatment. This second randomization involved all the patients in one trial, thus yielding placebo-placebo, placebo-orlistat, orlistat-placebo, and orlistat-orlistat groups.[9] In the second trial the second randomization involved only the group treated with orlistat during the first year, yielding placebo-placebo, orlistat-placebo, and orlistat-orlistat groups.[11]*

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Table 1. Weight loss with orlistat: results of 1-year clinical trials

	META-ANALYSIS OF FIVE TRIALS (NONDIABETIC SUBJECTS)		TRIAL OF TYPE 2 DIABETIC SUBJECTS	
SUBJECTS WHO LOST WEIGHT AFTER RANDOMIZATION	120 MG OF ORLISTAT TID PLUS DIET (N = 1561)	PLACEBO PLUS DIET (N = 1119)	120 MG OF ORLISTAT TID PLUS DIET (N = 163)	PLACEBO PLUS DIET (N = 159)
>5% weight loss	45.3% (P<.001 vs placebo)	23.4%	30.2% (P<.001 vs placebo)	13.2%
>10% weight loss	20.2% (P<.001 vs placebo)	8.3%	9.3% (P not significant vs placebo)	4.4%

Data from the European Agency for the Evaluation of Medicinal Products.[7]

in the orlistat group (120 mg tid) than in the placebo group (20.2% vs 8.3%, P<.001) (**Table 1**[7]), but did not differ significantly from that in the group treated with 60 mg of orlistat tid.

In the trial involving diabetic patients, the proportion of subjects who lost 5% or more of their initial weight was 13.2% in the placebo group and 30.2% in the orlistat group (P<.001). There was no statistically significant difference between groups in the proportion of patients who lost 10% or more of their initial weight (**Table 1**).[7]

A retrospective analysis of trial data identified factors predicting the efficacy of orlistat. Patients who lost at least 10% of their initial weight after 12 months of treatment were considered responders. According to this analysis, 90% of patients who lost less than 2.5 kg in the first 4 weeks of dietary intervention (ie, before orlistat therapy) were nonresponders, while 48% of patients who lost more than 5% of their initial weight during 12 weeks of treatment with orlistat and dietary intervention were responders.[7]

Orlistat is effective only if it is taken after the weight-loss phase. A double-blind trial involving subjects who had already lost weight by dieting compared the effect of 1 year of treatment with orlistat plus a stabilizing diet with that of placebo and the same diet. Patients regained a mean of 3.0 kg on orlistat (120 mg tid) and 5.1 kg on placebo (no published statistical analysis).[7]

A published trial with a second randomization phase after 1 year of treatment and 2 years of follow up treated patients with a low-calorie diet and placebo during the first year and randomized them to dietary intervention and orlistat or dietary intervention and placebo during the second year. Those given orlistat lost weight while those receiving placebo for 2 consecutive years regained weight: the difference between the two groups was 3.6 kg (P<.001).[9]

In short, orlistat treatment after the weight-loss phase at least partly avoids weight resumption in patients on a stabilizing diet.

Weight benefit after 1 year of treatment with orlistat and a low-calorie diet partly persists if orlistat is stopped and a stabilizing diet is started

Combined analysis of the two trials with a second randomization phase after a year of orlistat treatment showed that 53% of patients who received placebo during the second year regained 50% or more of the weight they initially lost.[9,11,12]

In these two trials the subjects treated for 2 years with orlistat (plus a low-calorie diet the first year and stabilizing diet the second year) regained significantly less weight during the second year than did subjects who stopped orlistat the second year (mean difference in weight gain +2.4 kg in each of these trials).[6,9,11] Mean weight of the latter patients showed a trend to joining that of the patients always receiving placebo, although it remained below their weight.[9]

Mild effect on plasma lipids. The effect of orlistat on lipid levels was studied in a meta-analysis, including the seven trials, that lasted at least 1 year.[7] After 1 year of treatment, total cholesterol and low-density lipoprotein cholesterol fell 0.3 mmol/L more in the dietary intervention plus orlistat groups (120 mg tid) than in the dietary intervention plus placebo groups, while high-density lipoprotein cholesterol increased 0.03 mmol/L less on average (P<.001 for each of the

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three comparisons). Statistical analysis showed that this effect was due solely to weight loss and not to a specific effect of orlistat.

Favourable effect on glycemic control in patients with type 2 diabetes. One trial compared orlistat with placebo for 1 year in obese patients (BMI 28 to 40 kg/m^2) with type 2 diabetes treated with a glucose-lowering sulfonamide, who were prescribed a low-calorie diet.[10]

In all, 163 patients received orlistat (120 mg tid), while 159 patients had placebo. On average, glycated hemoglobin levels fell by 0.28% between randomization and the end of treatment in the low-calorie diet plus orlistat group and increased by 0.18% in the low-calorie diet plus placebo group ($P < .001$).[8] Furthermore, 43.2% of the patients treated with orlistat were able to reduce their daily dose of glucose-lowering sulfonamide compared with only 28.9% of those given placebo (**e**).[10]

Frequent gastrointestinal effects; a question about breast cancer. We have little long-term experience with orlistat. The only available data on adverse effects come from clinical trials (with only a few thousand subjects) conducted before the drug was released onto the market, and there are currently no pharmacovigilance data (marketing authorization was granted in New Zealand only before the European Union). Because this is a new drug, and also a new therapeutic class, the utmost care must be taken (weak gastrointestinal absorption is no guarantee of safety).

Frequent gastrointestinal adverse effects. The mechanism of action of orlistat explains why, during the first year of clinical trials, an oily anal discharge was reported by 26.6% of patients receiving 120 mg tid (versus 1.3% of patients receiving placebo); 23.9% reported wind and uncontrolled flatulence (versus 1.4% of placebo patients); and 22.1% complained of defecation urgency (versus 6.7% of placebo patients).[7] Other gastrointestinal adverse events were diarrhea, soft stools, abdominal pain, and fecal incontinence (7.7% versus 0.9% of placebo patients).[7,9,12] The number of withdrawals during the first year of treatment, however, was higher in the placebo groups (35.3%) than in the groups receiving orlistat 120 mg tid (29.1%) (**f**). Adverse events were less frequent in the second year of treatment.[7,9]

Possible malabsorption of fat-soluble vitamins. In clinical trials, there was a slight but statistically significant reduction in plasma concentrations of vitamins D, E, and β-carotene.[7,9] Although no clinical consequences have been observed, the approved *Summary of Product Characteristics* (SPC) recommends a diet rich in vegetables and fruit and perhaps vitamin supplementation (to be taken some time before or after orlistat intake).

No major drug interactions have been reported with orlistat (**g**).

Worrying increase in breast cancer in clinical trials. Twelve cases of breast cancer were diagnosed during or after clinical trials in women treated with orlistat, compared with only two cases in women

e—*Criteria warranting dose reduction of the glucose-lowering sulfonamide were defined prospectively by the protocol.*
f—*In clinical trials, the number of withdrawals for adverse effects was higher in the orlistat groups, and the number of withdrawals for treatment failure was higher in the placebo groups.*[7]

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receiving placebo.[6,7] No mechanism has been identified to explain this statistically significant increase in the frequency of breast cancer (**h**). No carcinogenic effect was observed in animal studies.[6]

A bias linked to weight loss (that might have favoured tumour detection) was ruled out.[6,7] It was also argued that the tumours discovered in some of these patients were very probably present before the beginning of treatment with orlistat.[6,7] Nevertheless, an effect of the drug on tumour growth cannot be ruled out.[6] This potential risk was not mentioned in the SPC (European Public Assessment Report) approved by the European Medicines Evaluation Agency (**i**).[7]

In practice: limited utility; many outstanding questions

Sustained weight loss among obese patients requires long-term management lasting several years. Management is based on a low-calorie diet and increased physical activity, and possibly behavioural interventions. Drugs currently have only a limited role.

Weight loss attributable to orlistat after a year of treatment is moderate (3.5 kg on average), and treatment is costly. There are not enough data to guarantee the efficacy or safety of orlistat beyond 2 years of treatment. The long-term effect on body weight after orlistat withdrawal is not known.

It remains to be shown that orlistat has a tangible effect on morbidity or mortality, the main end point for all treatments of obesity. Only a moderate effect on some cardiovascular risk factors has been documented.

Orlistat might not be devoid of serious toxicity. In clinical trials, there was a statistically significant increase in risk of breast cancer in groups treated with this drug, and there is currently no reason to believe it was a simple artifact.

Despite the restrictions mentioned in the SPC, there is a danger of off-licence prescription. In particular, prescribers might be tempted to try this drug for patients in whom dietary measures fail, even though orlistat has no demonstrated efficacy in such cases.

The decision of the European Medicines Evaluation Agency to license this drug was not unanimous. Some members considered the effects of orlistat too small to be clinically relevant, while the risks, particularly that of breast cancer, remain uncertain.[7] According to our review of the clinical file, this minority opinion was justified from a public health viewpoint. And was it so urgent to authorize the drug? Why did the agency not wait until results of the ongoing Swedish study were obtained, especially since the protocol specifically provides for an assessment of the risk of breast cancer (**i**)[7]? Finally, why does the European SPC not mention the potential risk of breast cancer?

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Translated from *Rev Prescr* 1999;19(194):243-9. *Spelling, syntax, and reference style have been adapted to Canadian standards.*

g—*Potential interactions between orlistat and the following drugs have been tested: digoxin, phenytoin, warfarin, glibenclamide, nifedipine, furosemide, captopril, atenolol, pravastatin, and combined contraceptives. In studies involving healthy volunteers, orlistat did not significantly modify the pharmacokinetics of these drugs, with the exception of pravastatin, whose bioavailability was increased. The possible clinical consequences of this interaction are unknown.*[5,13-19]

h—*Various statistical analyses were done to estimate the degree of this risk. Their results were sometimes contradictory, and not all concluded that the risk is statistically significant. In an analysis undertaken by the US Food and Drug Administration, the comparison between the groups receiving placebo and receiving orlistat (120 mg tid) gave an odds ratio of 4.3 and a* P *value of .04.*[6]

i—*A trial lasting 2 years and involving 3350 patients who undergo regular mammography is under way in Sweden. Results are expected to be published in 2000.*[7]

Update

By May 14, 1999, no further sound data were available. Regular visits to the Food and Drug Aministration (FDA) website gave no new data on the risk of breast cancer. Orlistat was eventually granted a licence in the United States at the end of April 1999. As in Europe, the American product monograph and package insert do not draw attention to the risk of breast cancer. The FDA letter (personal communication from James Bilstad, Director, Office of Drug Evaluation II, FDA, to Peggy Jack, Program Director, Drug and Regulatory Affairs, Hoffmann–La Roche. 3 p) that stipulates the licensing requirements to Hoffmann-La Roche does, however, require a monthly report of breast cancer cases occurring during and up to the end of ongoing trials. This reinforces the message in our paper: pending full results of trials now under way, caution is required when considering orlistat, whose benefit on morbidity and mortality has not been documented. We shall return to this product as soon as new data are released.

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Our literature search was based on prospective, continuous scrutiny of contents listings of the main international journals and Current Contents at the Prescrire library, and on reference texts in clinical pharmacology (*Martindale—the Extra Pharmacopoeia, Meylers Side Effects of Drugs*, etc). We also consulted CD-ROM versions of MEDLINE (1966 to January 1999), EMBASE/Excerpta Medica Drugs and Pharmacology (1991 to September 1998), Reactions (1983 to September 1998), and Cochrane (1999, issue 1). Produits Roche sent us various published documents and a monograph on their product. We have also used the European Public Assessment Report (EPAR) from the European Medicines Evaluation Agency (July 29, 1998) and reports of meetings of the FDA specialist committee on marketing authorization of orlistat in the United States.

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Prescrire

Orlistat

Gare à la précipitation!

RÉSUMÉ

- **Les traitements de l'obésité sont décevants. Aucun n'a démontré un effet sur la morbi-mortalité. Les traitements non médicamenteux sont mal évalués. Le maintien à long terme d'une perte de poids nécessite une prise en charge prolongée.**
- **L'orlistat (Xenical®, Produits Roche), un inhibiteur des lipases gastro-intestinales, est officiellement indiqué, en association à une alimentation hypocalorique, dans la prise en charge de l'obésité.**
- **Son dossier d'évaluation est assez étoffé et de bonne qualité méthodologique, tout au moins sur le critère "perte de poids".**
- **Lors d'essais à moyen terme (12 à 24 mois), l'orlistat, administré à la posologie de 120 mg trois fois par jour et associé à une intervention diététique, a démontré une action favorable, quoique modeste (3,5 kg en moyenne), sur la perte de poids.**
- **Aucun essai à plus long terme n'a été effectué.**
- **On ne sait pas si ce médicament a un effet sur la morbi-mortalité liée à l'obésité.**
- **Lors des essais cliniques, il a été constaté une augmentation de la fréquence des cancers du sein parmi les patientes traitées par orlistat. L'évaluation de ce risque éventuel fait actuellement l'objet d'un essai spécifique.**
- **Les effets indésirables digestifs de l'orlistat sont fréquents.**
- **Le coût du traitement, entièrement à la charge du patient, ne peut être négligé.**

LA RÉDACTION NE PEUT SE PRONONCER

Chez les patients obèses, on ne sait pas actuellement si l'orlistat (Xenical®) a ou non, en plus d'un effet modeste sur la perte de poids, des effets cliniques tangibles en termes de morbi-mortalité.

Il existe un doute quant au risque de cancer du sein lié à la prise de l'orlistat. Le rapport bénéfices-risques de l'orlistat ne peut donc être établi actuellement.

Orlistat

Gélules

- **120 mg d'orlistat par gélule**

Le coût pour une posologie dune gélule trois fois par jour pour un mois s'éleve entre 120$ et 150$ (sans les frais d'ordonnance). Ce renseignement est fourni par David Freeman, pharmacien à l'Orillia Soldiers Memorial Hospital en Ontario.

Indication officielle

«Xenical est indiqué en association à un régime modérément hypocalorique dans le traitement de l'obésité (indice de masse corporelle [IMC] ≥30 kg/m^2) ou du surpoids (IMC ≥28 kg/m^2) associé à des facteurs de risques». «L'orlistat ne doit pas être utilisé chez les enfants» (AMM européenne, selon la procédure centralisée).

Inhibiteur des lipases gastro-intestinales; "agent antiobésité"

L'obésité est un problème de santé publique, concernant 6% à 10% de la population adulte française[1]. D'après les études épidémiologiques (principalement américaines), l'obésité (IMC 30 kg/m^2 [a]) est statistiquement corrélée à une mortalité plus élevée, l'excès de décès observé correspondant principalement à des décès cardiovasculaires[2].

L'obésité peut créer une gène dans le confort de vie, mais c'est également un facteur de risque de morbidité, notamment cardiovasculaire. Sa prise en charge thérapeutique doit s'effectuer à long terme, voire à vie, dans le but de maintenir une stabilisation définitive du poids après une phase de réduction pondérale initiale[2].

Les traitements actuels de l'obésité sont décevants (lire le mini-dossier). Plus de 80% des sujets ayant perdu du poids le reprennent progressivement[2]. Aucune thérapeutique de l'obésité n'a démontré un effet bénéfique en termes de réduction de la mortalité

a—*L'indice de masse corporelle (IMC) ou indice de Quetelet (en anglais, body mass index (BMI)) est calculé ainsi: poids/(taille)^2; le poids exprimé en kilogrammes et la taille en mètres^2.*

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ou de la morbidité cardiovasculaire. Il a même été supposé que l'alternance de phases de réduction pondérale et de reprise de poids pourrait être à l'origine d'une augmentation de la mortalité[2].

Le critère principal de jugement d'une thérapeutique de l'obésité devrait être la réduction de la morbimortalité. La réduction pondérale ne devrait être qu'un critère de jugement secondaire.

Plusieurs médicaments ont été proposés comme traitements adjuvants dans l'obésité. Certains ont été retirés du marché français en raison de leurs effets indésirables[3]. D'autres ont conservé leur autorisation de mise sur le marché, mais le risque de toxicomanie et d'hypertension artérielle pulmonaire qu'ils présentent n'autorise pas leur emploi au long cours (anorexigènes amphétaminiques [b])[4]. Les autres n'ont pas d'activité clinique spécifique démontrée (mucilages, "anti-œdemateux" et "lipolytiques", phytothérapie, etc).

Dans ce contexte de forte attente de la part des patients et des prescripteurs, la mise sur le marché de l'orlistat (Xenical®, Produits Roche) représente-t-elle un progrès dans la prise en charge de l'obésité? Quels bénéfices peut-on en attendre en termes de réduction de la mortalité? En termes de réduction de la morbidité liée à l'obésité? En termes de réduction pondérale?

Une évaluation limitée à des critères de jugement secondaires

L'orlistat est un inhibiteur des lipases. Administré par voie orale, il est peu absorbé et agit en inhibant partiellement l'activité des lipases gastro-intestinales[5]. Aux posologies recommandées, lorsque l'alimentation contient environ 30% des calories sous forme de lipides, il entraîne une excrétion fécale quotidienne de l'ordre de 20 g de graisses non digérées et non absorbées, soit l'équivalent de 180 kcal[6].

Le dossier d'évaluation clinique de l'orlistat est de bonne qualité méthodologique. L'évaluation en termes de réduction pondérale est bien étoffée, de même que l'évaluation en termes de modification des facteurs de risque cardiovasculaire. En revanche, il ne comporte pas d'essai ayant comme critère de jugement la morbidité ou la mortalité liée à l'obésité.

Dose optimale: 120 mg aux trois repas? Quatre essais randomisés ont visé à déterminer la dose optimale d'orlistat. Ces essais ont duré de 2 à 6 mois et ont inclus au total 1 421 patients[7]. Le plus grand essai, et le seul à avoir évalué la posologie de 240 mg trois fois par jour (tid), a inclus 676 sujets d'IMC compris entre 28 et 43 kg/m^2 [8]. Dans cet essai, quatre doses d'orlistat (30 mg, 60 mg, 120 mg et 240 mg) administrées tid ont été comparées au placebo. Tous les sujets suivaient par ailleurs une alimentation visant à créer un déficit énergétique de 500 kcal par jour (alimentation "hypocalorique").

La durée de traitement a été de 24 semaines. L'analyse a été faite en intention de traiter. Le critère d'évaluation principal a été la perte de poids. Celle-ci a été statistiquement supérieure à celle observée sous placebo dans les groupes recevant, en plus du régime, de l'orlistat aux posologies de 60 mg, ou 120 mg, ou 240 mg tid. C'est dans le groupe recevant 120 mg d'orlistat tid qu'elle a été la plus importante[8]. Cependant, dans les essais ayant duré 1 an, 120 mg tid ne sont pas apparus plus efficaces que 60 mg tid.

Sept essais à moyen terme versus placebo. Au total, sept essais randomisés en double aveugle, de durée comprise entre 1 et 2 ans, ont été réalisés: quatre essais américains et trois essais multicentriques européens. Ils ont inclus au total 4 188 patients âgés de plus de 18 ans, dont l'IMC était compris entre 28 et 47 kg/m^2 (répartition selon le sexe non précisée)[7]. À ce jour, seuls trois de ces essais sont publiés en détail[9-11], mais nous avons eu accès aux méta-analyses de ces essais par le rapport de l'Agence européenne du médicament[7].

Tous ces essais ont été réalisés versus placebo. Aucun essai n'a comparé l'orlistat à un autre traitement de l'obésité.

La perte de poids a été le critère de jugement principal de tous ces essais. Les autres critères de jugement étaient l'évolution des facteurs de risque cardiovasculaire suivants: glycémie, lipides plasmatiques, pression artérielle.

Six des sept essais comportaient une phase préliminaire de régime "hypocalorique" associé au placebo durant 4 ou 5 semaines, afin de sélectionner uniquement les patients motivés et observants. La randomisation n'avait lieu qu'à l'issue de cette phase.

Quatre de ces essais ont duré 2 ans et ont comporté deux phases distinctes: pendant la première année, évaluation de l'effet de l'orlistat associé à une alimentation hypocalorique (inférieure à 1 500 kcal par jour, dont 30% sous forme de lipides) (phase de réduction pondérale); pendant la deuxième année, évaluation de l'orlistat associé à un régime de stabilisation sur le

b—*Les anorexigènes amphétaminiques actuellement commercialisés dans cette indication sont: l'amfépramone, le clobenzorex, le fenproporex. Ils ont le statut de médicaments à prescription restreinte.*

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Tableau 1. Effet sur la réduction pondérale du traitement par orlistat: résultats des essais cliniques à 1 an[7]

	MÉTA-ANALYSE DES CINQ ESSAIS RÉALISÉS CHEZ LES SUJETS NON DIABÉTIQUES		ESSAIS CHEZ LES DIABÉTIQUES DE TYPE 2	
	120 MG D'ORLISTAT TID + RÉGIME (N = 1561)	PLACEBO + RÉGIME (N = 1119)	120 MG D'ORLISTAT TID + RÈGIME (N = 163)	PLACEBO + RÉGIME (N = 159)
Proportion de sujets ayant obtenu une perte de poids ≥10% du poids à la randomisation	45,3% (p < .001 vs placebo)	23,4%	30,2% (p < .001 vs placebo)	13,2%
Proportion de sujets ayant obtenu une perte de poids ≥10% du poids à la randomisation	20,2% (p < .001 vs placebo)	8,3%	9,3% (Absence de différence statistiquement significative vs placebo)	4,4%

maintien de la perte de poids (deux essais), ou la réduction de la reprise pondérale (deux essais) (**c,d**). Les résultats obtenus au cours de la phase de réduction pondérale de ces quatre essais et d'un autre essai, d'une durée d'un an et conduit selon un protocole identique, ont fait l'objet d'une méta-analyse[7].

Un autre essai d'une durée d'un an a évalué l'effet de l'orlistat associé à une alimentation hypocalorique chez des patients diabétiques de type 2 (alias non-insulinodépendants) avec surpoids ou obésité[10].

Le septième essai a évalué l'effet d'un an de traitement par régime de stabilisation + orlistat sur la stabilisation pondérale, après une perte de poids obtenue précédemment seulement grâce à une alimentation hypocalorique suivie pendant 6 mois.

Une réduction pondérale pendant la première année de traitement, seulement chez certains sujets. Dans la méta-analyse des cinq essais étudiant l'effet de l'orlistat versus placebo (associés à une alimentation hypocalorique), la perte de poids entre la randomisation et la fin du douzième mois de traitement a été en moyenne de 6,1 kg dans les groupes recevant 120 mg d'orlistat tid, et de 2,6 kg dans les groupes recevant le placebo (p < 0,001; poids moyen lors de la randomisation: 97 kg)[7]. La proportion de patients ayant perdu 10% ou plus de leur poids initial est plus élevée dans le groupe orlistat (120 mg tid) que dans le groupe placebo (20,2% vs 8,3%, p < 0,001), mais non statistiquement différente de celle du groupe de patients traités par 60 mg d'orlistat tid (**tableau 1**[7]).

Dans l'essai réalisé chez des patients diabétiques, la proportion de patients ayant perdu, après la randomisation, 5% ou plus de leur poids initial est de 13,2% pour le groupe ayant reçu le placebo et 30,2% pour le groupe orlistat (p <0,001); mais il n'y a pas de différence statistiquement significative pour la proportion de patients ayant perdu 10% ou plus de leur poids initial (**tableau 1**)[7].

Une analyse a posteriori des essais cliniques a permis de faire ressortir des critères prédictifs de l'efficacité du traitement par orlistat. Les patients ayant perdu au moins 10% de leur poids initial après 12 mois de traitement ont été considérés comme répondeurs. D'après cette analyse, 90% des patients ayant perdu moins de 2,5 kg au cours des 4 premières semaines d'intervention diététique (c'est-à-dire avant la prise d'orlistat) ne sont pas répondeurs; 48% des patients ayant perdu plus de 5% de leur poids initial en

c—*La prescription diététique du régime de stabilisation a été effectuée ainsi: les patients ayant perdu moins de 3 kg les 2 derniers mois de la première année ne modifiaient pas leur alimentation; les patients ayant perdu 3 kg ou plus durant les 2 derniers mois de la première année devaient augmenter leur apport calorique quotidien de 200 à 300 kcal.*

d—*Pour évaluer l'effet de l'orlistat sur la réduction de la reprise pondérale, les deux essais concernés ont comporté une deuxième randomisation, au bout d'un an de traitement. La 2e randomisation a été effectuée pour tous les patients dans un essai: on a ainsi obtenu des groupes placebo puis placebo, placebo puis orlistat, orlistat puis placebo, orlistat puis orlistat[9]. Dans le second essai, la 2e randomisation n'a eu lieu que dans le groupe traité par orlistat durant la première année. On a ainsi obtenu des groupes placebo puis placebo, orlistat puis placebo, orlistat puis orlistat[11].*

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ont été des échecs, et qui sont exposés à des complications importantes du fait de leur obésité[1,4].

Plusieurs essais randomisés ont comparé l'effet de différents types d'intervention chirurgicale. Six essais sur sept ont montré qu'un court-circuit gastro-intestinal (technique dite "Roux-en-Y") est plus efficace que la gastroplastie[3]. La perte de poids à 1 an est habituellement de l'ordre de 45 à 65 kg avec le court-circuit gastro-intestinal, et de l'ordre de 30 à 35 kg avec la gastroplastie[3]. Certains sujets reprennent du poids à distance de l'intervention; 20% des patients ne perdent pas de poids[2].

Ces interventions exposent à des effets indésirables, parmi lesquels on peut citer des malabsorptions digestives (notamment de la vitamine B_{12}), des troubles digestifs ("dumping syndrome"), des lithiases vésiculaires, des modifications de l'humeur[1]. La mortalité immédiate de la chirurgie gastrique est inférieure à 1%, à condition que l'intervention soit réalisée par une équipe expérimentée[2]. La réalisation d'une chirurgie gastrique impose d'importantes modifications du comportement alimentaire pour diminuer les effets indésirables digestifs[2].

En somme

Les effets à court terme des traitements de l'obésité sont mal évalués (à l'exception de ceux de quelques médicaments). L'évaluation de leurs effets sur le poids à long terme est pauvre, et les quelques résultats disponibles sont souvent décevants. Aucune évaluation de l'effet de ces traitements sur la mortalité n'a été effectuée.

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12 semaines de traitement par orlistat + intervention diététique sont répondeurs[7]. Ces critères ont été repris dans le libellé de l'indication officielle.

L'orlistat est efficace s'il est pris seulement après la phase de réduction pondérale. Chez des sujets ayant précédemment perdu du poids grâce à une intervention diététique, un essai en double aveugle a comparé l'effet d'une année de traitement par orlistat associé à un régime de stabilisation à l'effet d'un placebo associé à ce même régime. La reprise de poids a été de 3,0 kg en moyenne chez les patients traités par orlistat 120 mg tid, et de 5,1 kg chez les patients ayant reçu le placebo (analyse statistique non publiée)[7].

Dans un essai publié, ayant une seconde randomisation après 1 an de traitement et un suivi de 2 ans, les patients traités par alimentation hypocalorique + placebo la première année et qui ont été randomisés dans le groupe intervention diététique + orlistat la deuxième année ont perdu du poids durant cette deuxième année; alors que les patients randomisés 2 années de suite dans le groupe intervention diététique + placebo ont repris du poids à la fin de la deuxième année: différence entre ces deux groupes de 3,6 kg, en faveur de l'orlistat; $p < 0{,}001$[9].

Au total, la prise d'orlistat après la phase de réduction pondérale permet d'éviter, au moins en partie, la reprise de poids après institution d'un régime de stabilisation.

Le bénéfice pondéral obtenu après un an de traitement par orlistat + régime hypocalorique persiste en partie si l'orlistat est arrêté et un régime de stabilisation institué

L'analyse groupée des deux essais ayant comporté une 2e randomisation après un an de traitement par orlistat montre que 53% des patients ayant reçu le placebo la deuxième année ont repris 50% ou plus du poids initialement perdu[9,11,12].

Dans ces deux essais, les sujets traités 2 ans de suite par orlistat (+ régime hypocalorique la première année et régime de stabilisation la deuxième année) ont repris statistiquement moins de poids durant la deuxième année que les sujets ayant interrompu l'orlistat la deuxième année (différence moyenne de

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reprise de poids: +2,4 kg dans chacun de ces essais)[6,9,11]. Le poids moyen de ces derniers est resté inférieur à celui des patients ayant toujours été sous placebo, mais tend à se rapprocher de ce dernier groupe[9].

Un effet modeste sur les paramètres lipidiques sanguins. L'effet du traitement par orlistat sur les paramètres lipidiques a été étudié et a fait l'objet d'une méta-analyse incluant les sept essais de durée supérieure ou égale à 1 an[7]. Après 1 an de traitement, dans les groupes intervention diététique + orlistat (120 mg tid), le cholestérol total et le LDL-cholestérol ont baissé en moyenne de 0,3 mmol/l de plus que dans les groupes intervention diététique + placebo; l'HDL-cholestérol a moins augmenté (différence moyenne 0,03 mmol/l) que dans les groupes intervention diététique + placebo ($p < 0,001$ pour chacune de ces trois comparaisons). L'analyse statistique montre que cet effet est dû uniquement à la perte de poids et non à un effet spécifique de l'orlistat.

Un effet favorable sur le contrôle glycémique des patients diabétiques de type 2. Chez des patients obèses diabétiques de type 2 traités par sulfamide hypoglycémiant, d'IMC compris entre 28 et 40 kg/m^2, et auxquels était prescrite une alimentation hypocalorique, un essai a comparé l'efficacité d'un traitement d'1 an par orlistat au placebo[10].

En tout, 163 patients ont reçu de l'orlistat à la dose de 120 mg tid; 159 patients ont reçu le placebo. En moyenne, l'hémoglobine glyquée a diminué de 0,28% entre la randomisation et la fin du traitement dans le groupe alimentation hypocalorique + orlistat et augmenté de 0,18% dans le groupe alimentation hypocalorique + placebo ($p < 0,001$)[8]. 43,2% des patients traités par orlistat ont pu diminuer la dose quotidienne de leur sulfamide hypoglycémiant, versus 28,9% dans le groupe placebo (**e**)[10].

Des effets indésirables digestifs fréquents; un risque de cancer du sein à mieux évaluer. On dispose actuellement de peu de recul sur l'utilisation de l'orlistat et les risques qui y sont liés. Les seules données disponibles viennent des essais cliniques préalables à la mise sur le marché, qui n'ont porté que sur quelques milliers de sujets, et il n'existe pas actuellement de données de pharmacovigilance (seule la Nouvelle-Zélande avait accordé une autorisation de mise sur le marché avant la communauté européenne). S'agissant d'un nouveau médicament, et qui plus est d'une nouvelle classe thérapeutique, il convient donc de rester vigilant (la faible absorption digestive n'est en aucun cas la garantie d'une sécurité accrue).

Des effets indésirables digestifs fréquents. Le mode d'action de l'orlistat explique que durant la première année des essais cliniques, 26,6% des patients en recevant 120 mg tid ont signalé des pertes huileuses par l'anus (versus 1,3% des patients recevant le placebo); 23,9% des flatulences et émissions intempestives de gaz (versus 1,4% des patients recevant le placebo); 22,1% des impériosités fécales (versus 6,7% des patients recevant le placebo)[7]. Les autres événements indésirables digestifs décrits sont des diarrhées, des selles molles, des douleurs abdominales, voire une incontinence fécale (7,7% versus 0,9% de patients recevant le placebo)[7,9,12]. Le nombre de sorties d'essai au cours de la première année de traitement a néanmoins été plus important dans les groupes recevant un placebo (35,3%) que dans les groupes recevant de l'orlistat 120 mg tid (29,1%) (**f**). Ces événements indésirables ont été moins fréquents au cours de la deuxième année de traitement[7,9].

Une possible malabsorption des vitamines liposolubles. Les essais cliniques effectués ont montré une diminution discrète, mais statistiquement significative, des taux plasmatiques des vitamines D, E, et du β-carotène[7,9]. Bien qu'aucune conséquence clinique de cette diminution n'ait été constatée, le résumé officiel

e—*Les critères conduisant à diminuer la dose de sulfamide hypoglycémiant étaient définis a priori par le protocole.*

f—*Au cours des essais cliniques, le nombre de sorties d'essai pour effet indésirable a été supérieur dans les groupes orlistat, et le nombre de sorties d'essai pour échec du traitement supérieur dans les groupes placebo*[7].

g—*Diverses interactions médicamenteuses possibles ont été testées: digoxine, phénytoïne, warfarine, glibenclamide, nifédipine, furosémide, captopril, aténolol, pravastatine, contraceptifs estroprogestatifs. Au cours d'études ayant inclus quelques volontaires sains, l'orlistat n'a pas entraîné de modification significative de la pharmacocinétique de ces médicaments, sauf pour la pravastatine dont la biodisponibilité est augmentée par l'administration concomitante d'orlistat. Les conséquences cliniques éventuelles de cette interaction sont inconnues*[5,13-19].

h—*Différentes analyses statistiques ont été réalisées pour estimer l'importance de ce risque. Leurs résultats sont parfois contradictoires, toutes ne concluant pas à l'existence d'un risque statistiquement significatif. Selon une analyse effectuée par la Food and Drug Administration américaine, la comparaison entre des groupes placebo et orlistat 120 mg x 3 aboutit à un odds ratio de 4,3 avec p = 0,04*[6].

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des caractéristiques du produit (RCP) de Xenical conseille une alimentation riche en légumes et en fruits, voire une supplémentation vitaminique (à prendre à distance de la prise d'orlistat).

Aucune interaction médicamenteuse majeure n'a été constatée avec l'orlistat (g).

Une augmentation préoccupante des cancers du sein constatée au cours des essais cliniques. Douze cas de cancer du sein ont été découverts (pendant ou après les essais cliniques) chez des femmes traitées par orlistat; seulement deux cas ont été découverts chez des femmes recevant un placebo[6,7]. Aucun mécanisme connu ne permet d'expliquer cette augmentation statistiquement significative de fréquence de cancer du sein (h). Aucun effet cancérigène n'avait été constaté dans les études animales[6].

Un biais lié à la perte de poids, qui aurait favorisé la détection des tumeurs, a été éliminé[6,7]. Il a été objecté que les tumeurs découvertes chez certaines de ces patientes étaient très probablement présentes avant le début du traitement par orlistat[6,7]. Néanmoins, un effet sur la croissance tumorale ne peut être exclu[6]. Ce risque éventuel n'a pas été mentionné dans le RCP (EPAR) établi par l'Agence européenne du médicament[7]. En revanche, il a conduit la Food and Drug Administration américaine à retarder la mise sur le marché aux États-Unis (i).

En pratique: une place limitée, beaucoup d'inconnues

La prise en charge des patients obèses, dans le but de réaliser une perte de poids durable, doit s'effectuer à long terme, durant plusieurs années. Cette prise en charge est basée sur un régime de restriction calorique associé à une augmentation de l'activité physique et éventuellement à des interventions comportementales (lire le mini-dossier). Les médicaments n'ont actuellement qu'un rôle adjuvant limité dans cette prise en charge.

La perte de poids attribuable à l'orlistat après 1 an de traitement est modeste (3,5 kg en moyenne), pour un investissement financier important uniquement à la charge du patient. Aucune donnée n'existe actuellement permettant de garantir l'efficacité et la sécurité d'un traitement de plus de 2 ans par orlistat. C'est pourquoi la durée de prescription est actuellement officiellement limitée à 2 ans. Le devenir à long terme du poids des patients après l'arrêt d'un traitement par orlistat n'est pas connu.

i—*Un essai d'une durée de 2 ans, ayant inclus 3 350 patients et comprenant une surveillance mammographique, est actuellement en cours en Suède. Ses résultats sont attendus pour l'année 2000*[7].

Est-il actuellement démontré que l'orlistat a un effet clinique tangible sur ce qui devrait être le critère de jugement principal d'un traitement de l'obésité: la morbi-mortalité? Non, il a été seulement mis en évidence quelques effets modestes de ce médicament sur certains facteurs de risque cardiovasculaire.

L'administration de l'orlistat est-elle dénuée de risques majeurs? Ce n'est pas certain. Au cours des essais cliniques, une augmentation du risque de cancer du sein a été mise en évidence dans les groupes traités par ce médicament. Rien ne prouve actuellement qu'il s'agit là d'un simple artéfact.

Malgré les restrictions figurant dans le RCP, des dérives de prescription restent possibles. En particulier, la tentation peut être grande de prescrire ce médicament à des patients résistant aux mesures diététiques, indication dans laquelle l'orlistat n'a pas d'efficacité démontrée.

La décision de l'Agence européenne du médicament de mettre ce médicament sur le marché n'a pas été prise à l'unanimité: certains membres, minoritaires, ont estimé que les effets de l'orlistat sont trop modestes pour avoir un impact clinique alors que les risques sont mal connus, notamment pour ce qui concerne le cancer du sein[7]. Selon notre analyse du dossier d'évaluation clinique, cette minorité a raison si on se place d'un point de vue de santé publique. Quelle urgence y avait-il à commercialiser l'orlistat? Ne pouvait-on pas attendre au moins les résultats, prévus pour l'an 2000, de l'étude suédoise en cours, dont le protocole prévoit d'évaluer le risque de cancer du sein (i)[7]? Pourquoi le RCP européen ne signale même pas le problème non résolu du risque de cancer du sein?

La vigilance reste nécessaire....

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Références

Notre recherche documentaire a reposé sur le suivi prospectif et continu des sommaires des principales revues internationales et des Current Contents mis en œuvre au sein du Centre de documentation Prescrire, ainsi que sur la consultation systématique d'ouvrages de référence en pharmacologie clinique (*Martindale The Extra Pharmacopoeia*, Meyler's Side Effects of Drugs, etc.). Nous avons consulté sur CD-Rom les bases de données MEDLINE (1966 à janvier 1999), EMBASE/Excerpta Medica Drugs and Pharmacology (1991 à septembre 1998), Reactions (1983 à septembre 1998) et Cochrane (1999, issue 1). Par ailleurs, les laboratoires Produits Roche nous ont communiqué divers documents publiés et une monographie sur leur produit. Nous avons également utilisé l'European Public Assessment Report (EPAR) mis à disposition par l'Agence européenne du médicament (29 juillet 1998) et les comptes rendus des réunions du comité de spécialistes de la FDA consacrés à la demande d'AMM de l'orlistat aux États-Unis d'Amérique.

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Actualité

Au 14 mai 1999, aucune information solide supplémentaire n'est disponible. La consultation régulière du site internet de la FDA ne fournit aucune donnée supplémentaire quant au risque de cancer du sein. L'autorisation de mise sur le marché américaine a finalement été accordée à l'orlistat (Xenical®) fin avril 1999. Comme en Europe, le RCP et la notice d'emballage américaine n'attirent pas l'attention sur le risque non écarté de cancer du sein. Seule la lettre* de la FDA qui précise à la firme Hoffmann-La Roche les conditions d'obtention de l'AMM souligne la nécessité de fournir un relevé mensuel des cas de cancer du sein diagnostiqués au cours des essais en cours, et ce jusqu'à la fin de ces essais[1].

En l'état actuel des informations rendues disponibles, nous maintenons en son intégralité le message général de notre article du mois d'avril: jusqu'à plus ample informé, c'est-à-dire dans l'attente des résultats complets des essais en cours, la prudence s'impose vis-à-vis d'un traitement dont l'effet bénéfique sur la morbi-mortalité n'est pas démontré. Nous reviendrons bien entendu sur ce dossier quand nous aurons en mains des éléments nouveaux, cliniquement pertinents.

**Lettre de James Bilstad, Director, Office of Drug Evaluation II, FDA, à Peggy Jack, Program Director, Drug and Regulatory Affairs, Hoffmann–La Roche. 3 p.*

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Possible mechanisms involved in the vasorelaxant effect produced by clobenzorex in aortic segments of rats

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Abstract

Clobenzorex is a metabolic precursor of amphetamine indicated for the treatment of obesity. Amphetamines have been involved with cardiovascular side effects such as hypertension and pulmonary arterial hypertension. The aim of the present study was to investigate whether the direct application of 10^{-9}–10^{-5} M clobenzorex on isolated phenylephrine-precontracted rat aortic rings produces vascular effects, and if so, what mechanisms may be involved. Clobenzorex produced an immediate concentration-dependent vasorelaxant effect at the higher concentrations ($10^{-7.5}$–10^{-5} M). The present outcome was not modified by 10^{-6} M atropine (an antagonist of muscarinic acetylcholine receptors), 3.1×10^{-7} M glibenclamide (an ATP-sensitive K^+ channel blocker), 10^{-3} M 4-aminopyridine (4-AP; a voltage-activated K^+ channel blocker), 10^{-5} M indomethacin (a prostaglandin synthesis inhibitor), 10^{-5} M clotrimazole (a cytochrome P450 inhibitor) or 10^{-5} M cycloheximide (a general protein synthesis inhibitor). Contrarily, the clobenzorex-induced vasorelaxation was significantly attenuated ($P < 0.05$) by 10^{-5} M L-NAME (a direct inhibitor of nitric oxide synthase), 10^{-7} M ODQ (an inhibitor of nitric oxide-sensitive guanylyl cyclase), 10^{-6} M KT 5823 (an inhibitor of protein kinase G), 10^{-2} M TEA (a Ca^{2+}-activated K^+ channel blocker and non-specific voltage-activated K^+ channel blocker) and 10^{-7} M apamin plus 10^{-7} M charybdotoxin (blockers of small- and large-conductance Ca^{2+}-activated K^+ channels, respectively), and was blocked by 8×10^{-2} M potassium (a high concentration) and removal of the vascular endothelium. These results suggest that the direct vasorelaxant effect by clobenzorex on phenylephrine-precontracted rat aortic rings involved stimulation of the NO/cGMP/PKG/Ca^{2+}-activated K^+ channel pathway.

Key words: Clobenzorex; Rat aorta; Vasorelaxation; NO-cGMP; PKG pathway; K^+ channels

Introduction

Clobenzorex, N-(2-clorobenzyl)-amphetamine is an appetite suppressant indicated for the treatment of obesity, available in many countries (e.g., Mexico) as a prescription drug. Its hepatic metabolism leads to the synthesis of: i) conjugated metabolites of clobenzorex or p-hydroxyclobenzorex; ii) amphetamine, p-hydroxyamphetamine or conjugated metabolites of p-hydroxyamphetamine, and iii) hippuric acid. Clobenzorex is one of several drugs metabolized to amphetamine and excreted in the urine (1,2). Amphetamines used in weight management, such as sibutramine, fenfluramine and dexfenfluramine, have been involved with cardiovascular side effects such as hypertension and pulmonary arterial hypertension (3–5).

The cardiovascular side effects produced by amphetamines have been associated with an increased efflux of noradrenaline from the synaptic vesicles of sympathetic neurons, which interacts with vascular adrenoceptors such as α_1-adrenoceptors to produce vasoconstriction (6,7). However, there is evidence suggesting that the direct application of amphetamines on isolated rat aortic rings produces a vasoconstrictor effect through a mechanism not involving α_1-adrenoceptors (8–10). On the other hand, it has been suggested that *N,N*-dimethyl-thioamphetamine, an amphetamine derivative, does not produce aortic contraction (11). Furthermore, recent evidence suggests that the direct application of fenproporex, a metabolic precursor to amphetamine, produces a vasorelaxant effect on phenylephrine precontracted aortic rings (12).

The present study aimed to analyze whether the direct application of clobenzorex, a metabolic precursor

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of amphetamine, produces vascular effects on rat aortic rings, and if so, what mechanisms may be involved.

Material and Methods

Animals

Experiments were performed on isolated thoracic aortic rings of adult male Wistar rats (weighing 250–300 g; n=52), purchased from the bioterium of the Higher School of Medicine of the National Polytechnic Institute (Mexico City). Animals were housed in plastic cages in a special temperature-controlled room (22 ± 2°C, 50% humidity) on a 12:12 h light/dark cycle (lights on at 7:00 am). The study was approved by the Animal Care Committee of the Higher School of Medicine and the protocol was in agreement with the 1986 Animals (Scientific Procedures) Act of the British Parliament: http://www.legislation.gov.uk/ukpga/1986/14/contents (accessed on July 27, 2016).

Preparation of aortic rings

Animals were euthanized by decapitation and the aortas were immediately excised and placed in cold buffer, cleaned and freed from surrounding connective tissue. The isolated arteries were cut into rings (4–5 mm long) and placed in 10 mL tissue chambers filled with Krebs-Henseleit bicarbonate buffer (1.18×10^{-1} M NaCl; 4.7×10^{-3} M KCl; 1.2×10^{-3} M KH_2PO_4; 1.2×10^{-3} M $MgSO_4 \cdot 7H_2O$; 2.5×10^{-3} M $CaCl_2 \cdot 2H_2O$; 2.5×10^{-2} M $NaHCO_3$; 1.17×10^{-2} M dextrose, and 2.6×10^{-5} M calcium disodium EDTA). In some experiments, the concentration of KCl was increased to 8×10^{-2} M and that of Na^+ decreased to maintain osmotic equilibrium. Tissue baths, maintained at 37°C and pH 7.4, were bubbled with a mixture of 95% O_2 and 5% CO_2.

Aortic rings were mounted on two stainless steel hooks, one fixed to the bottom of the chamber and the other to a BIOPAC TSD125C-50 g force transducer connected to a BIOPAC MP100A-CE data acquisition system (BIOPAC Systems, Inc., USA) in order to record the isometric tension. Optimal tension, selected from preliminary experiments, was that which gave the greatest response to 10^{-6} M phenylephrine. The rings were given 2 g (100%) of initial tension and allowed to equilibrate for 2 h. Thirty minutes after setting up the organ bath, tissues were contracted with 10^{-6} M phenylephrine to test their contractile responses.

Endothelium-denuded aortic strips were prepared by turning the rings gently several times on the distal portion of small forceps. Endothelial integrity was pharmacologically assessed with acetylcholine-induced vasodilatation (10^{-6} M). Segments showing no relaxation to acetylcholine were considered to be endothelium-denuded. After exposure to 10^{-6} M phenylephrine or 10^{-6} M acetylcholine, tissues were rinsed three times with Krebs solution to restore basal tension.

Drugs

All drugs except clobenzorex were purchased from Sigma-Aldrich Co. (USA). Clobenzorex was a gift from Productos Medix, S.A. de C.V. Clobenzorex (Mexico), sodium nitroprusside (SNP), atropine, L-NAME, glibenclamide, 4-aminopyridine (4-AP), tetraethylammonium (TEA), clotrimazole and cycloheximide were dissolved in distilled water. Solutions of 10^{-5} M ODQ, 10^{-4} M KT 5823, 10^{-5} M apamin plus 10^{-5} M charybdotoxin and 10^{-3} M indomethacin were prepared by using 1.39 M dimethyl sulfoxide, 1.01 M ethyl acetate, 1.73 M acetic acid and 9.4×10^{-3} M sodium bicarbonate, respectively. Fresh solutions were made for each experiment.

Experimental protocol

To determine the mechanisms involved in the relaxant effect induced by clobenzorex on phenylephrine-precontracted rat aortic rings, two main sets of experiments were performed.

First set of experiments. Thirty minutes after restoration of basal tension, 10^{-6} M phenylephrine was added to rat aortic rings with or without endothelium. Sixty minutes later, after phenylephrine-induced contraction plateaued, clobenzorex and SNP began to be cumulatively added (10^{-9}–10^{-5} M and 10^{-11}–10^{-5} M, respectively) at intervals of around 5–6 and 3–4 min, respectively. Tension is reported as a percentage of the phenylephrine-induced contraction (4.02 ± 0.11 g = 100% for endothelium-intact rat aortic rings and 4.39 ± 0.17 g = 100% for endothelium-denuded rings).

Second set of experiments. Thirty minutes after adding 10^{-6} M phenylephrine (see first set of experiments), aortic rings with intact endothelium were preincubated for 30 min with one (or two) of various compounds in order to explore the mechanisms involved in the vasorelaxant effect produced by clobenzorex. The compounds used for preincubation were: i) 10^{-6} M atropine, a competitive muscarinic acetylcholine receptor antagonist; ii) 10^{-5} M L-NAME, a direct inhibitor of NO synthase; iii) 10^{-7} M ODQ, an inhibitor of nitric oxide-sensitive guanylyl cyclase; iv) 10^{-6} M KT 5823, an inhibitor of protein kinase G; v) 3.1×10^{-7} M glibenclamide, an ATP-sensitive K^+ channel blocker; vi) 10^{-3} M 4-aminopyridine (4-AP), a voltage-activated K^+ channel blocker; vii) 10^{-2} M TEA, a Ca^{2+}-activated K^+ channel blocker and nonspecific voltage-activated K^+ channel blocker; viii) 10^{-7} M apamin plus 10^{-7} M charybdotoxin, blockers of small- and large-conductance Ca^{2+}-activated K^+ channels, respectively; ix) 10^{-5} M indomethacin, a prostaglandin synthesis inhibitor; x) 10^{-5} M clotrimazole, a cytochrome P450 inhibitor; xi) 10^{-5} M cycloheximide, a general protein synthesis inhibitor; xii) distilled water (vehicle of atropine, L-NAME, 4-AP, TEA, clotrimazole and cycloheximide), xiii) 1.39×10^{-2} M dimethyl sulfoxide (vehicle of ODQ), xiv) 1.01×10^{-2} M ethyl acetate (vehicle of KT 5823), xv) 1.73×10^{-2} M

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acetic acid (vehicle of apamin plus charybdotoxin), or xvi) $9.4x10^{-5}$ M sodium bicarbonate (vehicle of indomethacin). Subsequently, clobenzorex was cumulatively added (10^{-9}–10^{-5} M) at intervals of around 4 min. Once reaching the desired concentration, the vasorelaxant response of the rings was assessed. In this way, the influence of the vehicles and drugs on the vasorelaxant response to 10^{-9}–10^{-5} M clobenzorex was tested.

Data analysis and statistics

Data are reported as means ± SE. In all experiments, n equals the number of animals from which aortic segments were obtained (8 in each case). Values of maximal vasorelaxation (E_{max}) were analyzed by using the Student's *t*-test. Effects of inhibitors/blockers on the vasorelaxant responses produced by clobenzorex on phenylephrine-precontracted aortic segments were analyzed by using a two-way analysis of variance. Each analysis of variance was followed by a Student-Newman-Keul's *post hoc* test. Statistical significance was considered at $P<0.05$ (13). The statistical analysis was performed in the SigmaPlot 12 program (Systat Software Inc., USA).

Results

Effect of clobenzorex on endothelium-intact and -denuded phenylephrine-precontracted rat aortic rings

Figure 1A–D shows typical traces of the effect produced by the *in vitro* application of clobenzorex and SNP in endothelium-intact and endothelium-denuded phenylephrine-precontracted rat aortic rings. The addition of phenylephrine to endothelium-intact and endothelium-denuded rat aortic rings produced a sustained contraction. The cumulative addition of clobenzorex produced a concentration-dependent vasorelaxant response in endothelium-intact (Figure 1A), but not in endothelium-denuded (Figure 1B) phenylephrine-precontracted rat aortic rings. The cumulative addition of SNP produced a concentration-dependent vasorelaxant response in both endothelium-intact (Figure 1C) and endothelium-denuded (Figure 1D) phenylephrine-precontracted rat aortic rings. The E_{max} values presented a significant difference ($P<0.05$) when comparing the effects of clobenzorex in endothelium-intact and -denuded phenylephrine-precontracted rat aortic rings: 110.07 ± 2.69 *vs* 5.49 ± 0.82% for clobenzorex and 106.12 ± 2.54 *vs* 104.12 ± 1.38% for SNP. EC_{50} values in endothelium-intact phenylephrine-precontracted rat aortic rings were $10^{-6.307}$ M for clobenzorex and $10^{-7.436}$ M for SNP.

Effect of atropine on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings

When comparing the effects of the absence and presence of atropine on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings, the E_{max} values were not significantly different: 92.18 ± 2.82 *vs* 94.00 ± 2.55, respectively (Figure 2).

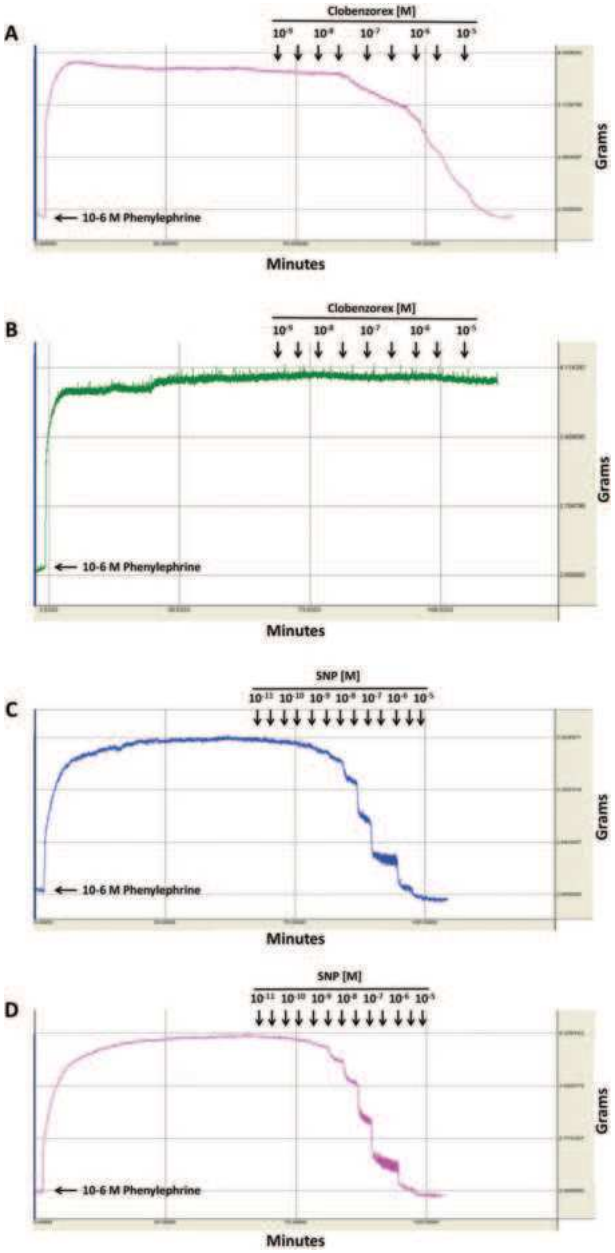

Figure 1. Representative tracings illustrating the relaxation response produced by the application of clobenzorex and sodium nitroprusside on endothelium-intact and endothelium-denuded phenylephrine-precontracted rat aortic rings. Clobenzorex (*A*) and sodium nitroprusside (SNP) (*C*) produced a dose-dependent vasorelaxant response in endothelium-intact phenylephrine-precontracted rat aortic rings. Endothelial denudation blocked the vasorelaxation to clobenzorex (*B*), but not to SNP (*D*). Similar results were obtained in all assays (n=8).

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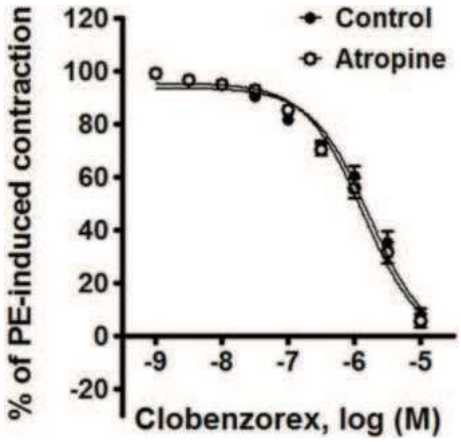

Figure 2. Pre-incubation with 10^{-6} M atropine did not modify the vasorelaxation produced by 10^{-9}–10^{-5} M clobenzorex in phenylephrine (PE)-precontracted rat aortic rings. Data are reported as means ± SE of 8 observations.

Effect of L-NAME, ODQ and KT 5823 on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings

When comparing the effect of the absence and presence of L-NAME, ODQ, and KT 5823 on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings, the E_{max} presented a significant difference ($P < 0.05$) in each case: 117.40 ± 1.31 *vs* 19.12 ± 4.41% for L-NAME, 111.48 ± 4.50 *vs* 8.34 ± 1.48%* for ODQ, and 95.77 ± 2.94 *vs* 10.82 ± 2.42% for KT 5823 (Figure 3).

Effect of glibenclamide, 4-AP, TEA, and apamin plus charybdotoxin on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings

When comparing the effect of the absence and presence of glibenclamide, 4-AP, TEA, and apamin plus charybdotoxin on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings, the E_{max} presented a significant difference ($P < 0.05$) only in the latter two cases: 116.19 ± 1.63 *vs* 108.26 ± 4.24% for glibenclamide, 108.98 ± 4.49 *vs* 109.97 ± 4.38% for 4-AP, 113.28 ± 2.74 *vs* 19.07 ± 2.80% for TEA, and 107.43 ± 5.24 *vs* 6.49 ± 1.22% for apamin plus charybdotoxin (Figure 4).

Effect of indomethacin, clotrimazole and cycloheximide on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings

When comparing the effect of the absence and presence of indomethacin, clotrimazole, and cycloheximide on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings, the E_{max} were not significant in any case: 103.16 ± 3.52 *vs* 100.23 ± 5.29% for indomethacin, 118.18 ± 2.45 *vs* 117.36 ± 2.45% for clotrimazole, and 102.94 ± 5.50 *vs* 100.88 ± 4.32% for cycloheximide (Figure 5).

Effect of distilled water, dimethyl sulfoxide, ethyl acetate, acetic acid and sodium bicarbonate on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings

When comparing the effect of the absence and presence of the different vehicles on the vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings, the difference in the E_{max} was not significant in any case (Table 1).

Discussion

The acute application of clobenzorex produced an immediate concentration-dependent vasorelaxant effect on endothelium-intact but not on endothelium-denuded phenylephrine-precontracted rat aortic rings. The effect was statistically significant at higher concentrations ($10^{-7.5}$–10^{-5} M) of this appetite suppressant drug. The present results suggest that, by itself, clobenzorex, a metabolic precursor of amphetamine, produces an endothelium-dependent vasorelaxant effect. In this sense, the endothelium-dependent vasorelaxant effect of clobenzorex was reinforced by the results found with SNP (a nitric oxide donor drug). The latter compound, used as a positive control of endothelium-independent vasorelaxation, produced a concentration-dependent vasorelaxant effect on both endothelium-intact and -denuded phenylephrine-precontracted rat aortic rings, as previously reported (14,15).

On the other hand, since the vehicle did not produce a concentration-dependent vasorelaxant effect in phenylephrine-precontracted rat aortic rings (data not shown), it can be ruled out that the clobenzorex-induced vasorelaxation was due to tachyphylactic effects caused by the repeated application of saline to aortic segments.

Clobenzorex produced a moderate vasodilator effect in the absence of phenylephrine-induced contraction. Nevertheless, we decided to perform our experimental protocol in rat aortic rings precontracted with phenylephrine to make evident the vasorelaxant effects produced by this appetite suppressant drug.

It is known that in the vasculature, the endothelial stimulation of muscarinic M_1, M_3, and M_5 receptors produces a vasorelaxant effect (16,17). However, the fact that atropine, an antagonist of muscarinic acetylcholine receptors (18), did not modify the direct vasorelaxation produced by clobenzorex on rat aortic segments excludes the possible involvement of stimulation of muscarinic acetylcholine receptors in the vasodilator responses produced by this appetite suppressant drug.

The vasorelaxant effect produced by clobenzorex was significantly attenuated by L-NAME (a direct inhibitor of

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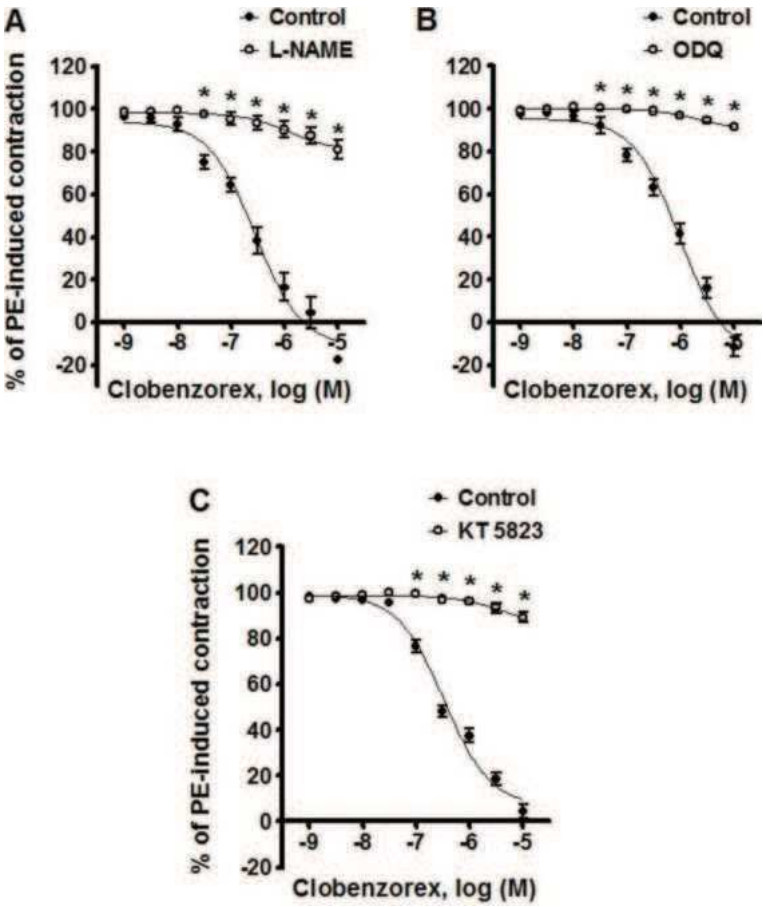

Figure 3. Vasorelaxation produced by 10^{-9}–10^{-5} M clobenzorex in phenylephrine (PE)-precontracted rat aortic rings. Assays were carried out to test the effect of: *A*, 10^{-5} M L-NAME; *B*, 10^{-7} M ODQ; and *C*, 10^{-6} M KT 5823. Data are reported as means ± SE of 8 observations. *$P < 0.05$ *vs* control (two-way ANOVA).

NOS) (19), ODQ (an inhibitor of nitric oxide-sensitive guanylyl cyclase) (20), and KT 5823 (an inhibitor of protein kinase G) (21). Hence, the stimulation of the NO-cGMP-PKG pathway is implied in the effect of this drug. In this sense, there is evidence that suggests that amphetamine can increase the NO synthesis in neurons of the striatal brain region through activation of NMDA receptors (22). This increase in the NO synthesis has been involved with stimulation of M_1 muscarinic acetylcholine receptors (23). However, the above evidence contrasts with the present study performed in rat aorta, in which, under the current experimental conditions, it was not possible to stimulate NMDA receptors. Moreover, since atropine did not modify the vasorelaxant effect produced by clobenzorex, it can be excluded the stimulation of muscarinic acetylcholine receptors. Indeed, we have no clear-cut explanation for the attenuating effect produced by L-NAME, ODQ and KT 5823. Possibly, clobenzorex enhances the activity or expression of the endothelial nitric oxide synthase producing the stimulation of the NO-cGMP-PKG pathway. However, this idea is still speculative and requires additional experiments that are beyond the scope of the present study.

On the other hand, the fact that the vasorelaxant effect produced by clobenzorex was unaffected by the respective vehicles of the L-NAME, ODQ and KT 5823 (distilled water, 1.39×10^{-2} M dimethyl sulfoxide and 1.01×10^{-2} M ethyl acetate) excludes the possibility that the attenuation of vasorelaxation produced by L-NAME, ODQ and KT 5823 was due to tachyphylactic effects induced by these vehicles.

The fact that the vasorelaxant effect produced by clobenzorex was unaffected by glibenclamide (an ATP-sensitive K^+ channel blocker) (24) and 4-AP (a voltage-activated K^+ channel blocker) (25,26), but significantly attenuated by TEA (a Ca^{2+}-activated K^+ channel blocker and non-specific voltage-activated K^+ channel blocker) (25,27) and apamin plus charybdotoxin (blockers of small- and large-conductance Ca^{2+}-activated K^+ channels, respectively) (28–30) suggests the involvement of Ca^{2+}-activated K^+ channels in the aforementioned effect. Furthermore, the vasorelaxant effect induced by clobenzorex was unaffected by distilled water (vehicle of L-NAME, 4-AP and TEA) and 1.73×10^{-2} M acetic acid (vehicle of apamin plus charybdotoxin). These results indicate that the vasorelaxation caused by clobenzorex is highly

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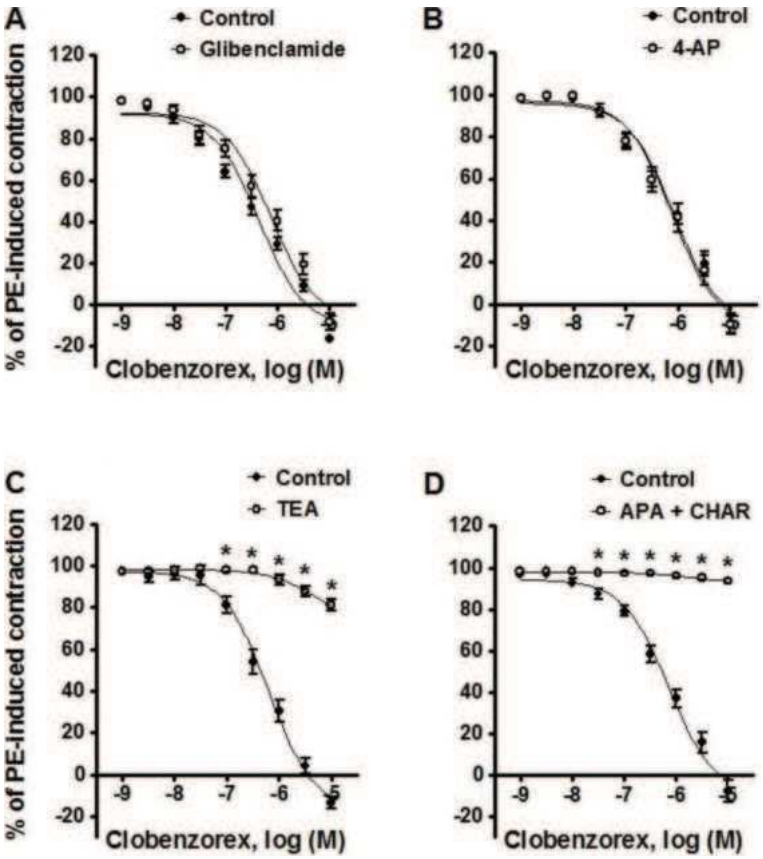

Figure 4. Vasorelaxation produced by 10^{-9}–10^{-5} M clobenzorex in phenylephrine (PE)-precontracted rat aortic rings. Assays were carried out to test the effect of: *A*, 3.1×10^{-7} M glibenclamide; *B*, 10^{-3} M 4-aminopyridine (4-AP); *C*, 10^{-2} M tetraethylammonium (TEA); and *D*, 10^{-7} M apamin (APA) plus 10^{-7} M charybdotoxin (CHAR). Data are reported as means ± SE of 8 observations. *$P < 0.05$ *vs* control (two-way ANOVA).

reproducible and rule out the possibility that attenuation produced by either of the K^+ channel blockers is due to tachyphylactic effects induced by their respective vehicles.

On the other hand, the combination of apamin plus charybdotoxin was used because it was previously reported that a complete blockage of Ca^{2+}-activated K^+ channels is necessary to produce a pharmacological response (29–31). In this sense, the two pilot experiments conducted in our laboratory showed that neither apamin nor charybdotoxin alone modified the vasorelaxant effect produced by clobenzorex (data not shown). Admittedly, we have no clear explanation about these observations, in which the combination of apamin plus charybdotoxin was necessary to block the vasorelaxant effect produced by clobenzorex. However, the experiments performed to elucidate whether the endothelium-derived hyperpolarizing factor EDHF could play a role in the vasorelaxant effect to clobenzorex, as previously reported for acetylcholine (32,33), are discussed below.

It has been suggested that additional mechanisms are involved in the endothelial control of vascular tone, such as prostacyclins (34) and EDHF, a cytochrome P450-derived arachidonic acid metabolite (35,36). However, the fact that the vasorelaxation produced by clobenzorex was unaffected by indomethacin, a prostaglandin synthesis inhibitor (37), clotrimazole, a cytochrome P450 inhibitor (36) and cycloheximide, a general protein synthesis inhibitor (38) excludes the involvement of prostacyclins, EDHF and protein synthesis in the endothelium-mediated vasorelaxation under the current experimental conditions. In this sense, the lack of effect of clotrimazole supports previous studies that suggest that EDHF plays no role in the endothelium-dependent relaxation in the rat aorta (39). Moreover, it must be emphasized that the concentration of cycloheximide used presently was high enough to block the protein synthesis (31).

The present study shows that the direct and acute *in vitro* application of clobenzorex to rat aortic rings produces an endothelium-dependent vasorelaxant effect. However, the *in vitro* character of this study represents a limitation. Although the current findings suggest a direct vasorelaxant effect of clobenzorex, *in vivo* studies are needed to establish whether the systemic administration of this appetite suppressant drug produces a vasodepressor effect. Moreover, clobenzorex is clinically used in the chronic treatment of obesity. However, the experiments of the present study were performed to analyze the effect of the direct application of clobenzorex in aortic

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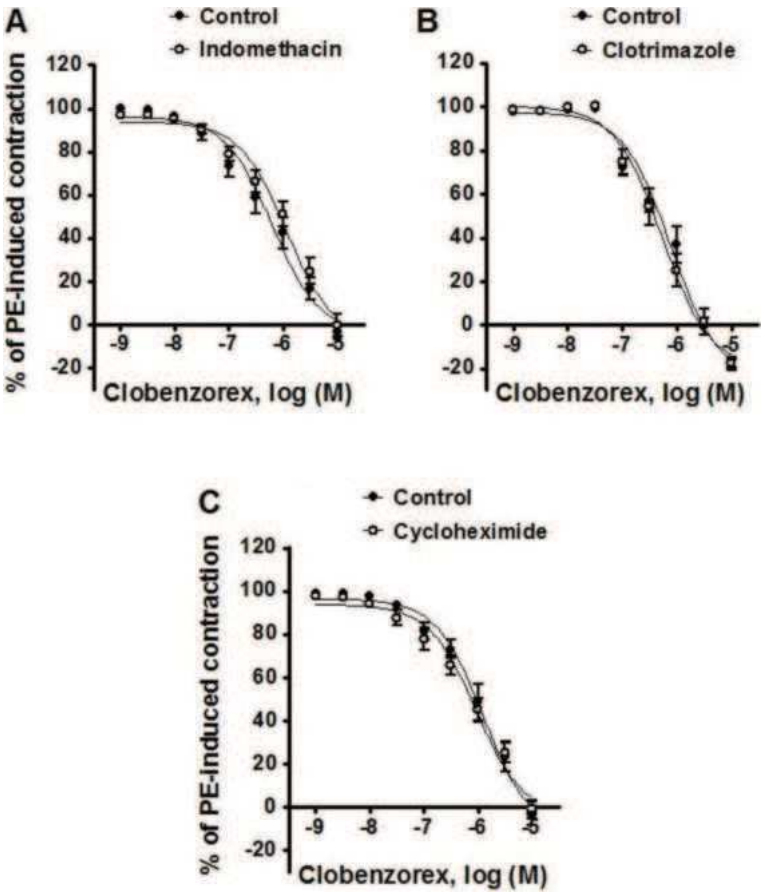

Figure 5. Vasorelaxation produced by 10^{-9}–10^{-5} M clobenzorex in phenylephrine-precontracted rat aortic rings. Assays were carried out to test the effect of: *A*, 10^{-5} M indomethacin; *B*, 10^{-5} M clotrimazole; and *C*, 10^{-5} M cycloheximide. Data are reported as means ± SE of 8 observations.

Table 1. Effect of the absence and presence of the vehicles on the maximal vasorelaxation induced by clobenzorex in phenylephrine-precontracted rat aortic rings.

Vehicle	Maximal vasorelaxation to clobenzorex (E_{max})	
	Absence	Presence
Distilled water	99.55 ± 1.39%	99.97 ± 1.68%
1.39×10^{-2} M dimethyl sulfoxide	100.91 ± 2.48%	101.78 ± 5.66%
1.01×10^{-2} M ethyl acetate	98.81 ± 3.25%	96.72 ± 3.01%
1.73×10^{-2} M acetic acid	112.12 ± 2.89%	113.52 ± 1.17%
9.4×10^{-5} M sodium bicarbonate	109.37 ± 5.62%	111.86 ± 3.24%

Data are reported as means ± SE of 8 observations.

segments of rats with normal weight. Admittedly, further experiments are needed to analyze the vascular effects produced by clobenzorex in obese animals. Overall, the present results suggest that the NO/cGMP/PKG/Ca^{2+}-activated K^+ channel pathway was a possible mechanism for the vasorelaxant effect observed.

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Review

Possible Mechanisms Involved in the Vasorelaxant Effect Produced by Anorexigenic Drugs in Rat Aortic Rings

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Abstract: Anorexigenics are compounds capable of reducing or suppressing appetite. Their three main types act on different neurotransmitters, either norepinephrine, serotonin or a combination of both. Among the drugs that act on norepinephrine are fenproporex, amfepramone and clobenzorex. Derivatives of the thyroid hormone triiodothyronine have also been associated with weight loss and used as a controversial treatment for obesity, despite their known cardiovascular side effects. Recent data suggest a possible vasodilating effect for these four substances that might be beneficial in a subset of patients. Herein we performed a systematic review of the literature (with emphasis on recent reports) to determine the implications and mechanisms of the vasodilating effects of some anorectics, specifically fenproporex, clobenzorex, amfepramone and triiodothyronine. Data analysis showed these four drugs to be vasodilating agents for rat aortic rings. The different mechanisms of action include endothelium-dependent vasodilation via activation of the NO-cGMP-PKG pathway and the opening of calcium-activated potassium channels. The finding of vasodilating activity indicates a potential role for some anorexigenic drugs in the treatment of obesity in hypertensive patients. Further in vivo studies are needed to test the clinical benefits of these four drugs.

Keywords: anorexigenics; obesity therapy; weight reduction; vasodilation; clobenzorex; fenproporex; amfepramone; triiodothyronine (T_3)

1. Introduction

Nowadays, obesity represents a very serious public health problem, the prevalence of which is considered a pandemic of the 21st century. It is defined as a systemic, chronic and metabolic disorder associated with cardiovascular disease, diabetes, hypertension, dyslipidemia and a diminished life expectancy [1,2].

Among the multiple treatments available to manage obesity, lifestyle changes and exercise are considered the cornerstone. Nevertheless, many obese and overweight patients also benefit from pharmacological therapy. Indeed, the clinical guidelines of the Endocrine Society recommend the inclusion of diet and exercise in all regimens focused on weight loss, as well as pharmacotherapy for patients with a body mass index (BMI) $\geq$27 in case of presenting any comorbidity, and all of those with a BMI > 30 [3]. Consequently, there are a wide variety of drugs currently available for treating obesity, such as pancreatic lipase inhibitors, thermogenic agents and hunger suppressors [2,4]. Regarding appetite suppressors, some anorexigenic drugs serve this function. The three main types of

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anorexigenics act on different neurotransmitters, either norepinephrine, serotonin or a combination of both.

Noradrenergic anorexigenic drugs are derived from amphetamines, which decrease or suppress appetite by increasing the release of catecholamines in the paraventricular nucleus of the hypothalamus. Amphetamines were withdrawn from the market because of being potentially addictive. New modified drugs have been developed with less risk of addiction, such as clobenzorex, fenproporex, mazindol, amfepramone and phentermine [5–8]. Nowadays, the latter is one of the most frequently used drugs for the management of obesity. Although phentermine was previously thought to have an addictive effect with long-term therapy; this was recently proven to be a misunderstanding [9–12].

Serotoninergic drugs are classified either as agonists or selective serotonin reuptake inhibitors (SRIs). Whereas the former trigger the release serotonin by its receptor, SRIs (e.g., fluoxetine and paroxetine) augment the extracellular concentration of this neurotransmitter by inhibiting its reuptake. The latter, usually prescribed for depression and other psychiatric disorders, are also helpful for promoting weight loss in the short term [5,7,8].

Finally, among the noradrenergic-serotoninergic drugs is a tertiary amine known as sibutramine. By acting through active metabolites that bind to the adrenergic α1, β1 and serotoninergic 2a and 2c receptors at the central level, it favors early satiety, stimulates thermogenesis and boosts energy expenditure, while showing low addictive capacity [5,7,13].

Another drug administered for the treatment of overweight and obesity is orlistat, an inhibitor of pancreatic lipases. Until 2012, it was the only anti-obesity drug to be approved for long-term use. This drug should be complemented with multivitamins, since it can reduce the absorption of liposoluble vitamins [14]. One study demonstrated beneficial results from the long-term intake of orlistat together with modifications in lifestyle (diet and physical activity). Over a one-year period, this combination led to significant weight loss (compared to the placebo) [15]. Recently, triiodothyronine (T_3) or thyroid hormone derivatives have been sold and consumed illicitly to achieve weight loss, producing some cases of thyrotoxicosis or serious cardiac problems [14].

The following is a systematic review of the effects of a subgroup of anorexigenic drugs (e.g., amfepramone, T_3, fenproporex and clobenzorex), including their vasodilator effect on rat aortic rings pre-contracted with phenylephrine.

2. Clobenzorex

Clobenzorex is well absorbed by the digestive tract. Its metabolism affords conjugated derivatives of clobenzorex, parahydroxyamphetamine, amphetamine and hippuric acid. Excreted in bile as conjugates of clobenzorex and parahydroxyamphetamine, it undergoes a more or less partial enterohepatic cycle. The fecal elimination of clobenzorex consists of unchanged products, conjugated derivatives, urinary parahydroxyclobenzorex (1.5–6% of the administered dose) and amphetamine (15%).

Among the adverse effects of clobenzorex are the consequences of vasoconstriction, including arterial hypertension and pulmonary artery hypertension. Vasoconstriction results from the increased flow of norepinephrine in the synaptic vesicles of sympathetic neurons, giving rise to the interaction of this neurotransmitter with α-1 receptors. Apart from hypertension, severe adverse events include polyuria, severe headaches and palpitations. Moderate side effects consist of depression, diarrhea and vomiting, while mild effects are irritability, dysuria, dry mouth and temporary anxiety [2,11,16–19].

A recent study by López et al. [17] showed that the acute application of clobenzorex induced an immediate concentration-dependent vasorelaxant effect on endothelium-intact but not on endothelium-denuded phenylephrine-precontracted rat aortic rings. The vasorelaxant effect produced by clobenzorex was significantly attenuated by L-NAME (NG-nitro-l-arginine methyl ester a direct inhibitor of nitric oxide synthase), ODQ (an inhibitor of nitric oxide-sensitive guanylyl cyclase), and KT 5823 (an inhibitor of protein kinase G). Hence, the stimulation of the NO-cGMP-PKG pathway is implied in the appetite suppressant effect. Whereas the vasorelaxant effect elicited by clobenzorex was unaffected by glibenclamide (an ATP-sensitive K^+ channel blocker) and 4-Aminopyridine (4-AP,

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a voltage-activated K^+ channel blocker), it was significantly attenuated by tetraethylammonium (TEA, a Ca^{2+}-activated K^+ channel blocker and non-specific voltage-activated K^+ channel blocker) and apamin plus charybdotoxin (blockers of small- and large-conductance Ca^{2+}-activated K^+ channels, respectively). This points to the involvement of Ca^{2+}-activated K^+ channels in the effect. Furthermore, the vasorelaxation produced by clobenzorex was unaffected by indomethacin (a prostaglandin synthesis inhibitor), clotrimazole (a cytochrome P450 inhibitor) and cycloheximide (a general protein synthesis inhibitor), thus excluding the participation of prostacyclins, endothelial-derived hyperpolarizing factor (EDHF) or protein synthesis in the endothelium-mediated vasorelaxation. Overall, these findings suggest that the NO/cGMP/PKG/Ca^{2+}-activated K^+ channel pathway is a plausible mechanism for the observed vasorelaxant effect [17].

3. Fenproporex

Another anorectic is fenproporex, chemically denoted as (±) N-2-cyanoethylamphetamine or (±) 3-[(1-methyl-2-phenethyl)amino]propionitrile. Since this drug has exhibited lipolytic action as well as effects in the hypothalamic nuclei that suppress appetite, it is used in the treatment of obesity and overweight.

Fenproporex is metabolized to amphetamine, which is a stimulator of the central and peripheral nervous system. In vivo studies on humans have shown two partially overlapping metabolic pathways for fenproporex: ring alteration by single and double aromatic hydroxylation followed by methylation and side chain degradation to amphetamine by *N*-dealkylation. The possible involvement of CYP2D6 in the formation of amphetamine from such precursor drugs is still controversial. Some authors have tried to explain the large differences in the amount of amphetamine metabolically formed from each of these precursors in function of CYP2D6 polymorphism [20].

The maximal plasmatic concentration of fenproporex occurs 2–4 h post-administration and its effect lasts for 6–8 h. It has renal clearance and eliminates fully after 48 h [2,7,21,22]. Among the adverse events are glaucoma, pulmonary artery hypertension, vomiting, headaches, vertigo, tachycardia and insomnia [19]. It is contraindicated in patients suffering from psychiatric disorders, drug abuse, alcoholism, hypertension, coronary artery disease and pulmonary hypertension [7,22,23].

According to recent reports, fenproporex induces a concentration-dependent relaxation on phenylephrine-precontracted rat aortic rings with functional endothelium, suggesting that its vasorelaxant effects are endothelium-dependent. The finding of a vasorelaxant effect elicited by fenproporex diverges from the vasoconstrictor effect of antiobesity drugs found in several in vitro and in vivo studies. Moreover, such a vasorelaxant response contrasts with the finding of increased plasma serotonin levels stemming from the intake of this drug, possibly implicating fenproporex in the development of acute cardiac and pulmonary disorders [24].

The authors propose that nitric oxide, K^+ channels and protein synthesis may participate in the vasorelaxant effects of fenproporex, because such effects are attenuated in endothelium intact rat aortic rings by the application of L-NAME, TEA (a non-specific voltage-activated K^+ channel blocker), and the combination of apamin and charybdotoxin or cycloheximide. Since glibenclamide did not modify vasorelaxant responses induced by fenproporex, K_{ATP} apparently is not involved in its vasorelaxant effects. It was concluded that fenproporex produces relaxant effects on phenylephrine-precontracted rat aortic rings, possibly by genomic mechanisms, the endothelial release of nitric oxide and/or stimulation of K_v-, and to a lesser extent through SK_{Ca}-, IK_{Ca}- and BK_{Ca}-channels [2].

4. Triiodothyronine

Thyroid hormones, prescribed to patients with thyroid gland dysfunction, can also be administered to obese euthyroid patients for weight loss. In several studies, the administration of thyroid hormone analogs to patients has led to a decrease in thyroid-stimulating hormone (TSH) and thyroxine (T_4). However, the results are inconsistent with respect to weight loss [25].

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Numerous experimental and clinical studies have demonstrated a relationship between thyroid hormones and the cardiovascular system [26]. There are various reports of a significantly modified cardiac function in patients with persistent subclinical thyroid dysfunction involving T_3 and/or thyroxine (T_4), which are thyroid hormones found in plasma and peripheral tissues, respectively. Triiodothyronine, the biological active thyroid hormone, is mostly generated by 5´-monodeiodination of T_4 in peripheral tissues [27].

Even though the findings described in many publications are contradictory, triiodothyronine has been illegally sold and consumed as a nutritional supplement in several countries. In recent years, a series of Hong Kong-based case studies reported that several patients suffered intoxication from the illegal use of this thyroid hormone, with a fraction presenting thyrotoxicosis and thyrotoxic periodic paralysis [28]. Among other adverse events are anxiety, hyperthyroidism, insomnia, diarrhea and palpitations [25].

An increase in the basal metabolic rate is the main effect of thyroid hormones, which explains why hypothyroid patients tend to be overweight and hyperthyroid patients frequently undergo weight loss [14]. Whereas most effects caused by thyroid hormones in bone and heart tissue are due to their interaction with the alpha isoform of thyroid receptors, the effects in the liver (e.g., a reduction in lipids) are mediated mainly by the β isoform of these receptors. Furthermore, the indiscriminate use of thyroid hormone analogs accelerates the loss of bone mass and can lead to arrythmias and cardiac hypertrophy if taken for prolonged periods of time [28,29].

Recent studies have focused on the cardiovascular effects of thyroid hormones, such as one that showed an acute concentration-dependent relaxant effect of triiodothyronine on vascular smooth muscle. This hormone generated a greater relaxant effect on aortic rings with than without endothelium. Its relaxant effect was partially inhibited in the absence of endothelium and in the presence of L-NAME, TEA, atropine (a competitive nonselective antagonist at central and peripheral muscarinic acetylcholine receptors), ODQ, KT 5823, and the combination of apamin and charybdotoxin or cycloheximide. The authors suggest the possible participation of the NO-cGMP-PKG pathway and/or Ca^{2+}-activated K^+ channels (via activation of muscarinic receptors) in the observed vasorelaxant effect [27].

5. Amfepramone

Amfepramone, also known as diethylpropion, is a sympathetic mimetic amine similar to amphetamines that stimulates the central nervous system and increases blood pressure. It is an appetite suppressing drug used for the treatment of obesity [7,22,30–32]. Monotherapy with this drug demonstrated greater efficacy than the placebo [23].

Amfepramone is rapidly and almost completely absorbed after oral administration. It undergoes significant metabolism in the liver, producing many active metabolites. Twelve non-acidic metabolites were identified, formed from three key reactions: N dealkylation, reduced ketone function, and benzene ring hydroxylation. The metabolites are conjugated upon the formation of hippuric acid and mandelic acid. Ninety percent of the absorbed product, including the metabolites, is eliminated in the urine within 30–40 h, at least 5% in the unmetabolized form. The rate of urinary excretion of the drug is a function of pH, decreasing with alkaline urine. The excretion half-life of the amfepramone-metabolite complex is approximately 10.4 h. In acidic urine, this parameter is diminished to 1.5–3 h. The plasma half-life of amfepramone is approximately two hours [33].

Among its adverse events are transitory ischemic attacks, pulmonary artery hypertension, [23] vomiting, anxiety, headaches, euphoria, insomnia, leukopenia and agranulocytosis [19]. It can cross the blood-brain barrier as well as enter the placenta and is contraindicated for hyperthyroidism, severe hypertension and glaucoma [7,11,22,23].

According to some in vitro studies, amfepramone causes vasodilatation in endothelium-intact rat aortic rings pre-contracted with phenylephrine, an effect not found after the mechanical removal of this tissue. Hence, the vasorelaxant effect of amfepramone is apparently endothelium-dependent. Overall, the evidence suggests that amfepramone may produce direct relaxation in systemic vasculature.

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However, its predominant effect following administration by a systemic and central route seems to be vasoconstriction, possibly due to the activation of central mechanisms.

The direct vasorelaxant effects on aortic rings generated by amfepramone reinforce previous findings from experiments carried out on dogs, in which the intravenous injection of amfepramone produced a dose-dependent depressor reaction [34] and a transient vasodepressor effect [35]. In contrast to these results are additional observations made in the same report. The intracerebroventricular administration of amfepramone in dogs elicited a marked pressor response [34] and the transient vasodepressor effect induced by the intravenous injection of amfepramone in dogs was followed by a vasopressor effect [35]. Moreover, vasorelaxant responses to the application of amfepramone in aortic rings do not coincide with one clinical study in which oral intake of amfepramone triggered ischemic attacks in the brain of an obese patient [36].

In the experiments conducted by Lopez-Canales et al. [31], the vasorelaxant response in rat aortic rings caused by amfepramone was unaffected by 4-AP and glibenclamide, while being significantly attenuated by L-NAME and TEA. This suggests the involvement of nitric oxide released by endothelium tissue and the activation of K^+ channels by Ca^{2+}, also supported by the fact that the mechanical removal of endothelium tissue blocked vasorelaxation. Additionally, the NO pathway and K^+ channels have been implicated in the endothelial-mediated control of vascular tone. Nevertheless, the vasorelaxation prompted by amfepramone was unaffected by indomethacin and by clotrimazole, excluding the participation of prostacyclins, the endothelium-derived hyperpolarizing factor in the endothelium-mediated vasodilation.

In summary, the data suggest that vasorelaxant responses produced on phenylephrine-precontracted rat aortic rings by amfepramone involve the activation of endothelial nitric oxide synthase and the opening of Ca^{2+}-activated K^+ channels [31].

6. Final Considerations

Due to the technological advances and lifestyle of the Western world, obesity has been on the rise for decades [1,14]. Although diet and exercise are the most important factors for achieving weight loss, for certain people this is either not enough or not possible. As a result, a wide variety of drugs have been developed to help with weight loss, each one with a different therapeutic target. Whereas some evoke satiety, others suppress the appetite or burn fat at an accelerated rate [14].

In general, anorexigenics have been related over the years to certain adverse effects, regardless of their family or type. Due to their vasoconstrictive mechanism of action, cardiovascular problems are among such effects [1,3,5,37]. That is, these drugs lead to elevated levels of catecholamines in the blood, which causes a rise in blood pressure. Since the risk factors faced by obese patients are aggravated, anorexigenics are contraindicated for the management of obese patients [1,37].

According to the findings of the present study, on the other hand, fenproporex [2], amfepramone [31], clobenzorex [17] and T_3 [27] administered under certain conditions do not increase blood pressure, but instead generate endothelium-dependent vasodilation via nitric oxide, potassium and voltage-dependent calcium channels. Consequently, these drugs could probably be used without adverse cardiovascular effects [2,17,27,31]. Further research is needed to explore the possible involvement of type M1 muscarinic receptors in the mechanism of action of the aforementioned drugs, a mechanism suggested in previous reports. In addition to clarifying their mechanisms of action, evaluation should be made of the potential of fenproporex, amfepramone, clobenzorex and T_3 as treatments for obesity, especially in patients suffering from comorbidities such as hypertension.

7. Conclusions

Although it is well-recognized that exercise and diet are the key factors in managing obesity or overweight, pharmacological treatments clearly play an important role under some circumstances. Some researchers have made the effort to find other pharmacological effects of anorectics distinct from the ones classically understood. These drugs have been contraindicated for obese and overweight

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patients due to their adverse effects, especially cardiovascular effects. Taking into consideration the vasodilator effect produced at the vascular-endothelial level by various anorectics, particularly fenproporex, amfepramone, clobenzorex and T_3, they could potentially gain a safer profile for use in obese patients with cardiovascular disease.

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Original Research Article

POSSIBLE PERIPHERAL LIPOLYTIC EFFECT OF CLOBENZOREX HYDROCHLORIDE IN PATIENTS WITH OBESITY

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Summary

Introduction. It is well known that clobenzorex hydrochloride is an amine that inhibits appetite by its sympathomimetic action on the lateral hypothalamus. However, it has been reported that this drug also has a peripheral lipolytic effect, which has not been studied sufficiently. The aim of this study was to evaluate such lipolytic action in obese patients. Methods: A controlled clinical trial was conducted with male and female patients having class I and II obesity and an age range of 30-60 years (n=33). Patients were randomized into two groups: the experimental group (n=16) received a single oral dose of 90 mg clobenzorex, and the control group (n=17) a placebo. Blood samples were taken at 0, 60, 120, 180 and 240 min post-administration to determine serum levels of free fatty acids (FFAs), glycerol, glucose, triglycerides, HDL-cholesterol and LDL-cholesterol. The atherogenic index (AI) was calculated. Results: Over the time of the study (0-240 min), the experimental group showed an increase in HDL-cholesterol (from 42.0 to 55.6 mg/dl; p=0.01), FFAs (from 0461 to 1009 mMol/l; p=0.01), and glycerol (from 0.123 to 0.170 mMol/lt; p=0.01). There was a significant decrease in AI (from 5.87 to 5.26; p=0.01). The placebo group showed a significant increase only in FFAs (from 0436 to 0824 mMol/lt; p=0.01) and a significant decrease in AI (from 4.51 to 4.05; p=0.01). Conclusion: These results suggest that clobenzorex hydrochloride has peripheral lipolytic activity, which can be considered as an additional effect, synergistic with its central pharmacological mechanism.

Keywords: clobenzorex hydrochloride, drug treatment, obesity.

Introduction

Clobenzorex hydrochloride is among the anti-obesity agents used in Mexico. This drug is a phenethylamine anorectic with sympathomimetic effects that act on the lateral hypothalamus, increasing the release of catecholamines that favor satiety and diminish the consumption of food, thus considerably reducing caloric intake. This drug is absorbed rapidly and completely 4-6 hours after being taken [1, 2]. It is recommended for patients with a BMI above 30 kg/m^2 that do not respond to diet and exercise [3]. In addition to its central

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action mechanism, it has been reported that clobenzorex hydrochloride has peripheral effects [4], including a lipolytic effect that may be another mechanism of this drug [5].

It is known that lipolysis is a catabolic process that leads to the decomposition of triglycerides stored in adipocytes and therefore to the release of fatty acids and glycerol [6, 7]. Recent studies have revealed that the metabolic pathway of lipolysis is not just stimulated by catecholamines and inhibited by insulin [8]. It also involves the participation of the endocrine system and regulation by the paracrine system, as well as the intervention of molecular mechanisms in the hydrolysis of triglycerides [3, 9].

Catecholamines modulate lipolysis through beta-adrenergic and alpha-2-adrenergic receptors. This action is due to the *in vivo* participation of these receptors in the physiological control of lipolysis in subcutaneous adipose tissue [10, 11]. However, the only report that evidences the lipolytic action of anorectics is that published in 1972 by Charbonnier. Hence, the aim of the present study was to evaluate lipolytic action of clobenzorex hydrochloride by determining the serum levels of free fatty acids (FFAs) and glycerol after the administration of a 90-mg dose. We hypothesized that the serum levels of free fatty acids (FFAs) and glycerol will be increase after administration of a 90mg of clobenzorex hydrochloride.

Material and Methods

Study design

A longitudinal, prospective study was carried out with control groups related to time. The participants in the study were randomly assigned to the treatment with clobenzorex hydrochloride or the placebo. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964, as revised in 2013 as well as the International Conference on Harmonized Tripartite Guideline for Good Clinical Practice and the Mexican norm. Informed consent was obtained from all patients.

Research subjects

There were 33 participants in the present study, 16 of whom received clobenzorex hydrochloride and 17 the placebo. The patients were 18-60 years old and had a BMI ≥30 kg/m^2, with class I or II obesity according to the classification of the World Health Organization. All were evaluated by elaborating the clinical history recommended by the Mexican Academy for the Study of Obesity, with the aim of discarding any possibly comorbidity related to obesity.

Treatment

The experimental group (n=16) received a single oral dose of 90 mg of clobenzorex hydrochloride after a fast of 12 hours, while the control group (n=17) received an oral dose of the placebo under the same conditions.

Anthropometric measurements

These parameters were evaluated according to the guidelines established by the manual of the International Society for the Advance of Kineanthropometry (ISAK, 2006). Weight was measured with a physician's scale (SECA 700, Spain) after a 12-hour fast and evacuation of the bladder, placing the individual in an upright position with arms hanging loosely to the side and without movement. The BMI was calculated with the Quetelet formula. The waistline and hip were measured with an anthropometric flexible plastic tape measure (Hergom-Mexico). The individual was placed in the previously described upright position, but with arms crossed over the thorax. To calculate the waist-hip ratio (WHR), the waistline value was divided by the hip circumference.

Biochemical measurements

A 5-ml blood sample was taken from each participant by puncture of the median cubital vein of the left arm. As aforementioned, the drug or placebo was administered after a 12-hour fast. Blood was taken at 0, 60, 120, 180

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and 240 min post-administration , placing samples in a vacutainer tube (K2E/KE EDTA, New York). Immediately afterwards, the blood was centrifuged with a clinical apparatus (SMART R17-Korea) at 4200 rpm for 10 min to obtain serum, which was frozen to -70 °C (freezer, MDF-U5486SC-USA/Canada) to await further processing.

Values were obtained for the concentration of glucose (Glu), total cholesterol (Chol), high density lipoprotein (cHDL), low density lipoprotein (cLDL), triglycerides (TGs), FFAs and glycerol. The atherogenic index (AI) was calculated by dividing total cholesterol by cholesterol bound to cHDL. Colorimetric enzyme analysis (GOD/PAP Randox, United Kingdom) was used to obtain values for glucose and TGs, taking a reading with a standard spectrophotometer (Microlab 200, Mexico). The wavelength was 546 nm for glucose and 505 nm for TGs, in each case using 5 µL of serum and 500 µL of the reactive, and comparing the results to a standard. Values for non-esterified fatty acids (NEFA) and glycerol were obtained by using spectrometry (VV2150-New York) and quantitative in vitro colorimetric enzyme analysis.

Statistical analysis

One-way analysis of variance (ANOVA) was performed, with a paired analysis for independent variables as well as multiple comparisons (MSD, minimum significant difference) and correlations, comparing the variables before and after the interventions. The SPSS version 19 program was used for all statistical analysis.

Results

Basal characteristics

All 33 participants had a BMI greater than 30 and less than 40 g/m2, with a comparable average age between the experimental and control groups (41.3 ± 12.5 vs. 38.7 ± 13.2, respectively; p = 0.57). Regarding gender, the group administered clobenzorex hydrochloride had 12 women and 4 men, while the placebo group had 12 women and 5 men. (Table I)

Table I. Anthropometric indicators of the clobenzorex versus placebo group by gender.

Indicators	Women		p*	Men		p*
	Clobenzorex n=12	Placebo n=12		Clobenzorex n=4	Placebo n=5	
BMI	31.6 ± 2.6	33.5 ± 3.9	t=-1.42, p=0.17	32.6 ± 3.4	33.7 ± 1.4	t=-0.66, p=0.55
Weight	77.7 ± 8.1	83.7 ± 8.1	t=-1.83, p=0.08	97.3 ± 18.8	102.2 ± 7.3	t=-0.5, p=0.65
Size	1.57 ± 0.05	1.58 ± 0.04	t=-0.92, p=0.37	1.72 ± 0.07	1.74 ± 0.05	t=-0.46, p=0.66
Waist	96 ± 5.3	96.7 ± 9.1	t=-0.25, p=0.81	106.5 ± 7.5	108.8 ± 3.3	t=-0.57, p=0.60
Hip	111.4 ± 6.3	116.6 ± 9.0	t=-1.61, p=0.12	109.3 ± 8.5	112 ± 3.1	t=-0.62, p=0.57
WHR	0.89 ± 0.07	0.83 ± 0.07	t=2.09, p=0.05	0.97 ± 0.04	0.98 ± 0.02	t=-0.44, p=0.68

Abbreviations: BMI – body mass index, WHR – waist-hip ratio; Women with the clobenzorex (n=12) and placebo (n=12) treatment. Men with the clobenzorex (n=4) and placebo (n=5) treatment. Values are expressed as the mean ± SE. *P<0.05 treatment vs placebo.

Biochemical variables

The initial values of the blood parameters showed no significant differences between groups (p>0.05), except that cLDL had a significantly higher value for the clobenzorex hydrochloride group (Table II; p=0.04).

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Table II. Values for the biochemical indicators for patients receiving a single 90 mg dose of clobenzorex (n=16) or the placebo (n=17).

Indicators	**Time (min)**					**p (ANOVA)**
	Basal	**60**	**120**	**180**	**240**	
Chol (mg/dl)						
Clobenzorex	209.6 ± 36.1	215 ± 43.5	215.8 ± 45.6	223.3 ± 45.2	217.2 ± 36.1	F=0.22, p=0.92
Placebo	201.1 ± 41.4	201.1 ± 47.5	211.2 ± 45.6	205.6 ± 37	205.1 ± 43.3	F=0.16, p=0.96
p (Student *t*)	t=0.63, p=0.53	t=0.88, p=0.39	t=0.29, p=0.77	t=1.23, p=0.23	t=0.87, p=0.39	
cHDL (mg/dl)						
Clobenzorex	42 ± 16.8	39.4 ± 12.5	40.5 ± 15.2	45.7 ± 14	55.6 ± 15.9	F=3.09, p=0.02
Placebo	47.8 ± 12.5	41 ± 15.3	43.7 ± 18.7	54 ± 33	53.7 ± 12.2	F=1.48, p=0.22
p (Student *t*)	t=-1.12, p=0.27	t=-0.33, p=0.75	t=-0.54, p=0.6	t=-0.95, p=0.35	t=0.37, p=0.71	
AI						
Clobenzorex	5.87 ± 2.86	5.97 ± 2.34	5.92 ± 2.2	5.26 ± 1.8	4.15 ± 1.14	F=36.0, p=0.01
Placebo	4.51 ± 1.65	5.64 ± 2.77	5.73 ± 3	4.52 ± 1.74	4.05 ± 1.52	F=48.4, p=0.01
p (Student *t*)	t=1.66, p=0.11	t=0.37, p=0.71	t=0.21, p=0.84	t=1.2, p=0.24	t=0.22, p=0.83	
cLDL (mg/dl)						
Clobenzorex	107.1 ± 30.6	101.6 ± 22.2	106.9 ± 33.4	101.7 ± 24.5	103.1 ± 26.3	F=0.16, p=0.96
Placebo	85.7 ± 25.3	84.9 ± 30.1	90.5 ± 28.1	92 ± 24.8	96.2 ± 33.8	F=0.45, p=0.77
p (Student *t*)	t=2.19, p=0.04	t=1.82, p=0.08	t=1.52, p=0.14	t=1.13, p=0.27	t=0.66, p=0.51	
Glu (mg/dl)						
Clobenzorex	63.7 ± 13.7	63.1 ± 8.4	59.1 ± 9.9	58.2 ± 9.9	57.3 ± 14	F=1.06, p=0.38
Placebo	61.6 ± 14.5	59.6 ± 12.3	60.7 ± 13.2	58.9 ± 9.8	59 ± 9.2	F=0.16, p=0.96
p (Student *t*)	t=0.44, p=0.67	t=0.95, p=0.35	t=-0.39, p=0.7	t=-0.19, p=0.85	t=-0.41, p=0.69	
TGs (mg/dl)						
Clobenzorex	147.8 ± 70	151.1 ± 74.8	146.3 ± 70.4	153.5 ± 72.6	162.2 ± 73.3	F=0.12, p=0.97
Placebo	129.8 ± 49.5	124.9 ± 50.6	123.7 ± 53.7	124.7 ± 48.4	125.7 ± 45.6	F=0.04, p=0.99
p (Student *t*)	t=0.85, p=0.4	t=1.17, p=0.25	t=1.03, p=0.31	t=1.33, p=0.19	t=1.71, p=0.10	
FFAs						

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(mMol/lt)						
Clobenzorex	0.461 ± 0.206	0.552 ± 0.187	0.73 ± 0.315	0.875 ± 0.365	1.009 ± 0.381	F=8.93, p=0.01
Placebo	0.436 ± 0.169	0.516 ± 0.185	0.564 ± 0.215	0.708 ± 0.265	0.824 ± 0.263	F=8.25, p=0.01
p (Student *t*)	t=0.37, p=0.72	t=0.55, p=0.59	t=1.76, p=0.09	t=1.5, p=0.15	t=1.61, p=0.12	
Glycerol (mMol/lt)						
Clobenzorex	0.123 ± 0.027	0.15 ± 0.064	0.16 ± 0.052	0.178 ± 0.055	0.17 ± 0.038	F=3.12, p=0.02
Placebo	0.12 ± 0.03	0.136 ± 0.039	0.129 ± 0.043	0.141 ± 0.036	0.138 ± 0.031	F=0.89, p=0.47
P	t=0.26, p=0.8	t=0.81, p=0.43	t=1.89, p=0.07	t=2.3, p=0.03	t=2.62, p=0.01	

Abbreviations: Chol – total cholesterol; cHDL – high-density lipoprotein; AI – atherogenic index, cLDL – low-density lipoprotein; Glu – glucose; TGs – triglycerides; FFAs – free fatty acids. Clobenzorex (n=16); PB – Placebo (n=17). Values are expressed as the mean ± SE. *P<0.05 treatment vs placebo.

For the FFAs, there was a significant increase from the basal to final measurement in both groups (Table II; p=0.01). Upon making the comparison between the differences in the basal and final state, there was a significant increase in favor of the clobenzorex hydrochloride group (Table III; p=0.05). The correlations between both groups were significant (Table III; p=0.01).

Table II. Correlations and differences between basal and final values of biochemical indicators: a single dose of 90 mg clobenzorex (n=16) versus placebo (n=17).

Indicators	**Difference: Basal-final**	**P-value for the difference: Basal-final**	**r-Pearson**
Chol (mg/dl)			
Clobenzorex	7.6 ± 26.3	t=-1.16, p=0.26	r=0.74, p=0.01
Placebo	4.0 ± 23.1	t=-0.72, p=0.48	r=0.85, p=0.01
p (Student *t*)	t=0.41, p=0.68		
cHDL (mg/dl)			
Clobenzorex	13.6 ± 12.2	t=-4.45, p=0.01	r=0.72, p=0.01
Placebo	5.9 ± 7.7	t=-3.15, p=0.01	r=0.8, p=0.01
p (Student *t*)	t=2.13, p=0.04		
AI			
Clobenzorex	-1.72 ± 2.21	t=3.11, p=0.01	r=0.71, p=0.01
Placebo	-0.46 ±	t=2.47, p=0.03	r=0.89,

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	0.77		p=0.01
p (Student *t*)	t=-2.16, p=0.04		
cLDL (mg/dl)			
Clobenzorex	-4 ± 18.9	t=0.84, p=0.41	r=0.79, p=0.01
Placebo	10.5 ± 21.1	t=-2.05, p=0.06	r=0.78, p=0.01
p (Student *t*)	t=-2.08, p=0.05		
Glu (mg/dl)			
Clobenzorex	-6.41 ± 17.5	t=1.47, p=0.	r=0.21, p=0.45
Placebo	-2.58 ± 14.5	t=0.73, p=0.47	r=0.32, p=0.21
p (Student *t*)	t=-0.68, p=0.5		
TGs (mg/dl)			
Clobenzorex	14.41 ± 34	t=-1.7, p=0.11	r=0.89, p=0.01
Placebo	-4.08 ± 24.1	t=0.7, p=0.5	r=0.87, p=0.01
p (Student *t*)	t=1.79, p=0.08		
FFAs (mMol/lt)			
Clobenzorex	0.548 ± 0.28	t=-7.84, p=0.01	r=0.7, p=0.01
Placebo	0.388 ± 0.142	t=-11.28, p=0.01	r=0.87, p=0.01
p (Student *t*)	t=2.06, p=0.05		
Glycerol (mMol/lt)			
Clobenzorex	0.047 ± 0.034	t=-5.65, p=0.01	r=0.51, p=0.04
Placebo	0.018 ± 0.023	t=-3.16, p=0.01	r=0.71, p=0.01
P	t=2.9, p=0.01		

Abbreviations: Chol – total cholesterol; cHDL – high-density lipoprotein; AI – atherogenic index, cLDL – low-density lipoprotein; Glu – glucose; TGs – triglycerides; FFAs – free fatty acids. Clobenzorex (n=16); PB – Placebo (n=17). Values are expressed as the mean ± SE. *P<0.05 treatment vs placebo.

Glycerol showed a statistically significant increase in the clobenzorex hydrochloride group from the basal to final value (Table II; p=0.02), while this increase was small and insignificant for the placebo group (Table II). The comparison of the differences between the basal and final value was significant for both groups (p=0.01), as were the correlations

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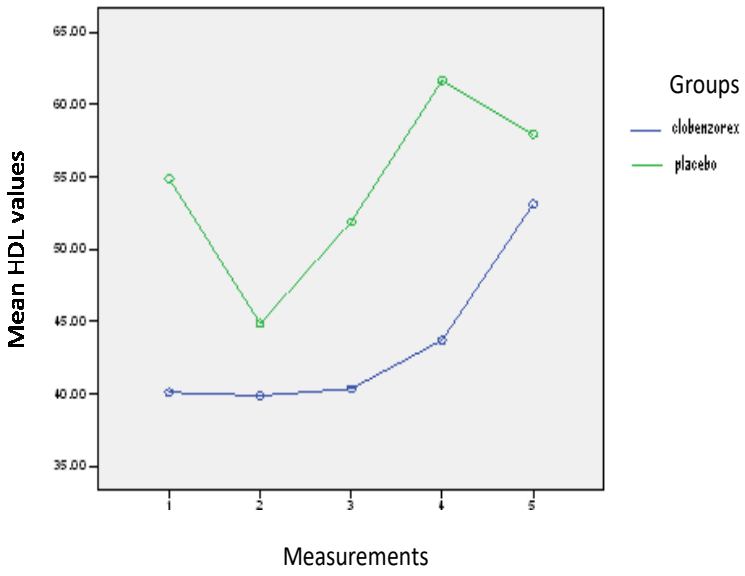
(Table III; p=0.01). The value for cHDL increased significantly during the study for the clobenzorex hydrochloride group (Table II; p=0.02), while the increase was small and insignificant for the placebo group. The difference between the basal and final value of this parameter was significant (p=0.04) when comparing the two groups, as was the correlation between the initial and final states (Table III; p=0.01).

The placebo group began with a higher average cHDL value than the clobenzorex hydrochloride group. In both groups, this value decreased at 60 min post-administration of the respective treatment, and then increased at 180 min. In the placebo group the level of cHDL stabilized between 180 and 240 min, while there was a significant increase during this lapse of time for the clobenzorex hydrochloride group (Graph 1).



Graph 1. *c-HDL values*
Concentration-time curve for cHDL in plasma (post-administration time in hours): Clobenzorex (n=16) vs placebo (n=17). *P<0.05.

The difference between the basal and final value was significant for AI in both groups (p=0.01), but was greater for the clobenzorex hydrochloride group (p=0.01 vs p=0.03), as was the correlation between the initial and final states (Table III; p= 0.01).

The value for cLDL decreased gradually and significantly during the study for the clobenzorex hydrochloride group, while increasing in the placebo group (Table II). This difference was significant for the former group (Table III; p<0.05). The correlations between both groups in the basal and final state were significant (Table III; p=0.01).

The correlations between the measured variables (without considering the particular time of measurement) is shown in Table IV for the clobenzorex hydrochloride group and Table V for the placebo group.

Since the anthropometric measurements were only taken at the basal state, correlations were established with the initial measurements of the biochemical indicators. In the clobenzorex hydrochloride group, the BMI and the WHR were inversely associated with cholesterol. For the placebo group, the important positive correlations were the

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BMI with TGs, the WHR with TGs, and FFAs with glycerol, while an inverse correlation was found for WHR with cHDL (Tables IV and V). or patients in the clobenzorex hydrochloride group, age directly influenced the values for CT, Glu and FFAs, while for patients in the placebo group age was positively associated with CT, Glu and TGs (Tables IV and V). Additionally, Chol was positively associated with FFAs in the clobenzorex hydrochloride group and with AI in the placebo group. The value of cHDL was not associated with Chol in either of the two groups in the study. In the clobenzorex hydrochloride group, an increase in cHDL was associated with a decrease in AI and TGs.

Table IV. Correlations (r-Pearson) between anthropometric and biochemical indicators in patients receiving clobenzorex.

Indicators		Chol	HDL	AI	LDL	Glu	TGs	FFAs	Glycerol
Weight	r	.306	-.230	-.282	.224	.465	.206	.262	-.172
	p	.249	.391	.289	.404	.069	.444	.327	.525
Size	r	.068	-.368	**-.513***	.361	.420	.131	.464	-.356
	p	.802	.161	**.042**	.170	.105	.629	.070	.176
BMI	r	**.548***	.021	.190	-.001	.396	.225	-.098	.273
	p	**.028**	.938	.480	.997	.129	.402	.719	.307
Waist	r	.211	-.187	-.141	.242	.445	.421	.080	-.103
	p	.433	.487	.604	.367	.085	.104	.767	.703
Hip	r	.229	-.016	.476	-.242	.155	.206	-.015	-.232
	p	.394	.953	.062	.366	.566	.444	.956	.387
WHR	r	**-.520***	-.345	-.272	.302	.048	.203	-.173	-.180
	p	**.039**	.190	.308	.256	.859	.452	.523	.504
Age	r	**.383***	-.01	.01	.16	**.215**	.03	**.279***	-.06
	p	**.01**	.95	.99	.17	**.05**	.79	**.01**	.64
Chol (mg/dl)	r		.06	.18	**.552***	-.09	.04	**.277***	.05
	p		.57	.14	**.01**	.41	.75	**.01**	.67
cHDL (mg/dl)	r			**-.241***	.02	-.05	**-.468***	.10	.19
	p			**.03**	.87	.65	**.01**	.39	.09
AI	r				.07	-.03	-.04	-.09	-.09
	p				.54	.81	.73	.44	.43
cLDL (mg/dl)	r					-.12	.12	.07	.02
	p					.30	.28	.56	.87
Glu (mg/dl)	r						**.235***	.04	-.09
	p						**.04**	.75	.43
TGs (mg/dl)	r							**.401***	.15
	p							**.01**	.18
FFAs (mMol/l)	r								**.564***
	p								**.01**
Glycerol (mMol/l)	r								
	p								

[1] In nmol/ml [2] In nmol/mg prot. p<0.05, significant

Abbreviations: Chol – total cholesterol; AI – atherogenic index, cHDL – high-density lipoprotein; cLDL – low-density lipoprotein; Glu – glucose; TGs – triglycerides; FFAs – free fatty acids. Clobenzorex.

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Table V. Correlations (r-Pearson) between anthropometric and biochemical indicators in patients given the placebo.

Indicators		Chol	cHDL	AI	cLDL	Glu	TGs	FFAs	Glycerol
Weight	r	-.181	**-.517***	.129	.222	.252	**.664****	**.754****	.306
	p	.488	**.034**	.622	.391	.330	**.004**	**.001**	.232
Size	r	.048	**-.622***	.184	.432	.497*	.417	**.761****	.065
	p	.856	**.008**	.479	.084	.042	.096	**.001**	.803
BMI	r	-.284	.001	-.065	-.174	-.237	**.478**	.163	.385
	p	.270	.999	.805	.505	.360	**.053**	.532	.127
Waist	r	-.082	-.303	.352	.184	.202	**.677****	.465	**.518***
	p	.755	.236	.166	.479	.437	**.003**	.060	**.033**
Hip	r	-.097	.352	.299	-.288	-.211	.092	-.333	-.030
	p	.710	.166	.243	.262	.417	.725	.192	.909
WHR	r	-.064	**-.561***	.159	.356	.313	**.564***	**.728****	**.521***
	p	.808	**.019**	.542	.160	.221	**.018**	**.001**	**.032**
Age	r	**.235***	0.02	.02	0.08	**.317***	**.245***	-0.03	0.10
	p	**0.03**	0.85	.83	0.49	**0.01**	**0.02**	0.77	0.35
Chol (mg/dl)	r		0.08	**.220***	**.501***	0.20	0.04	-0.08	0.19
	p		0.49	**.04**	**0.01**	0.07	0.71	0.45	0.09
cHDL (mg/dl)	r			-.206	0.19	-0.08	0.00	0.13	0.18
	p			.06	0.09	0.48	0.99	0.25	0.09
AI	r				-.04	.01	-.09	**-.256***	-.11
	p				.72	.92	.44	**.02**	.34
cLDL (mg/dl)	r					-0.07	0.09	0.09	**.276***
	p					0.54	0.42	0.44	**0.01**
Glu (mg/dl)	r						0.19	-0.02	**.314***
	p						0.08	0.87	**0.01**
TGs (mg/dl)	r							**.276***	**.274***
	p							**0.01**	**0.01**
FFAs (mMol/l)	r								**.539***
	p								**0.01**
Glycerol (mMol/l)	r								
	p								
	p								

[1] In nmol/ml [2] In nmol/mg prot. p<0.05, significant

Abbreviations: Chol – total cholesterol; cHDL – high-density lipoprotein; AI – atherogenic index, cLDL – low-density lipoprotein; Glu – glucose; TGs – triglycerides; FFAs – free fatty acids. Placebo.

Discussion

Obesity is associated with a 3-fold or greater increase in the risk of acute myocardial infarction [(12)]. The American Heart Association (AHA) has reclassified obesity as a modifiable risk factor for coronary heart disease (CHD). One of the main contributors to the morbidity of cardiovascular disease (CD) is an alteration in plasmatic lipids and the levels of lipoproteins [(13)]. Recent epidemiological data show that a low plasmatic level of cHDL also represents a risk factor for cardiovascular disease (CD) [(14)]. On the contrary, an increase of 1 mg/dl in the plasmatic level of cHDL is associated with a decrease in the risk of CHD by 2-3% [(15)].

A significant difference in cHDL was observed when comparing the difference

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between the basal and final states between groups, since the change in cHDL was significantly greater in the group treated with clobenzorex hydrochloride (p = 0.04). In this respect, some studies suggest that certain amines improve the profile of lipids, increasing the levels of cHDL [16, 17]. There are also studies suggesting that clobenzorex hydrochloride tends to increase the level of cHDL in the short run [3, 5, 18]. It has been reported that this increase in cHDL may be due to the fact that clobenzorex hydrochloride is a sympathomimetic amine that acts on the ventrolateral nucleus of the hypothalamus, prompting the release of noradrenaline and dopamine in the synaptic spaces, and consequently specific metabolic modifications (20).

On the other hand, there are studies that consider cLDL as one of the main indicators of risk for CD (19-22). Yet in the present study, there was no significant modification of this parameter in either group during the time of the experiment. However, there was a significant difference between the value found in the clobenzorex hydrochloride group and that observed in the placebo group ($p<0.05$), due to the gradual decrease in the former. The levels of cLDL cholesterol are significantly lower after the administration of sympathomimetic amines that act on the ventrolateral nucleus of the hypothalamus (23), which explains the gradual decrease in cLDL due to the treatment with clobenzorex hydrochloride. This same effect was also described by Charbonnier (5).

Multiple studies have shown that lipidic indexes, such as that represented by CT/c-HDL, are better predictors of the risk of CHD than the separate measurement of CT, cHDL, cLDL or TGs (24). It has been observed that the atherogenic index in particular is a good predictor of the intimal medial thickness of the common carotid artery, having a predictive power of this parameter as well as the development of atherosclerosis that is superior to any of the isolated variables (25). With respect to the atherogenic index in the present study, a greater reduction was shown for patients in the clobenzorex hydrochloride than placebo group (Table III; p=0.04).

Although a significant increase in FFAs was observed for both groups, this change was significantly greater for patients receiving clobenzorex hydrochloride than those given the placebo (Table IV). There was a clear tendency in the former group to a lineal increase in FFAs as time passed (from 0-240 min post-administration).

It is known that fasting stimulates lipolysis in an acute manner, as well as positively regulating the serum level of fatty acids and glycerol, which act as oxidative substrates to maintain the energy requirements for other metabolic tissues (26). Catecholamines are the primary activators of the lipolysis triggered by fasting (27-29), but this mechanism is altered in obese people (30). In the present study, there was a significantly greater rise in the concentration of FFAs in the clobenzorex hydrochloride versus placebo group, which can be explained by the effect that this anorectic has on beta-adrenergic receptors due to being a phenethylamine. By binding to this receptor, clobenzorex hydrochloride prompts the formation of cAMP, thus promoting the activation of the hormone-sensitive lipase (HSL) and consequently indirectly causing an increase in the mobilization of lipids through the release of endogenous catecholamines (10, 31, 32). The study done by Charbonnier in 1972 measured the variation in FFAs during 4 hours of fasting, observing only a slight increase that did not reach statistical significance (5). In the present study, the rise in FFAs was significant when comparing the value of this parameter between the clobenzorex hydrochloride and placebo group (Table IV), demonstrating that this medication accelerates the increase in FFAs by stimulating lipolysis in the periphery, possibly by the previously described mechanism.

Regarding the level of glycerol, the tendency in the clobenzorex hydrochloride group was for a constant rise from the basal to final measurement (Table III), resulting in a

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significant overall increase (p=0.02). This was not the case for the placebo group. It has been clearly established that a simultaneous increase in FFAs and glycerol is associated with lipolytic activity and an increase in the metabolism of fatty acids (33, 34). Pharmacological evidence suggests that dopaminergic mechanisms, which are found with clobenzorex hydrochloride, can also be involved in the control of thermogenesis (18, 20, 35), activating lipolysis in peripheral adipose tissue (21, 36) and promoting the release of fatty acids (37, 38).

The mobilization of lipids from fat reserves is an important part of providing for energy demand. In vitro studies have demonstrated the capacity of catecholamines to stimulate the mobilization of lipids from subcutaneous adipose tissue, a process which is found to be altered in obese individuals (39, 40). This is an important mechanism that contributes to the development of obesity, since catecholamines are the only hormones with a pronounced stimulatory effect on lipolysis in human fat cells (27, 41).

The correlations identified in the current contribution show that age does not directly influence the anthropometric variables in either of the two groups. However, age did indeed directly influence the values of FFAs, Chol and Glu in the patients receiving clobenzorex hydrochloride. The process of ageing is one of the factors that alters lipid metabolism (42). At a younger age in men and women, there is a greatly reduced risk of an adverse cardiovascular event. The reasons for the protection provided against CD in younger women and men are complex, but a significant contribution can be attributed to the greater levels of cHDL observed in younger people. Moreover, previous studies have demonstrated that there is a relation between age and an increase in Glu and FFAs (43).

Particularly important in the present study is the association between the increase in cHDL and the decrease in AI found only in the group treated with clobenzorex hydrochloride. An increase in cHDL levels has been associated with a decrease in the incidence and prevalence of adverse cardiovascular events (44). Many of the therapies aimed at promoting a rise in cHDL levels have not demonstrated a consistent clinical benefit (15). In the present study, this parameter was beneficial, having a direct effect on the atherogenic index in the patients receiving clobenzorex hydrochloride.

Conclusions

The present results suggest that clobenzorex hydrochloride has peripheral lipolytic activity, which could be considered as a pharmacological action for possible use in treating obesity and preventing the complications of this disease. According to the present results, it would be convenient to perform measurements of catecholamines in urine in order to identify whether there is a correlation between the changes that are shown in FFAs and glycerol, and in this way, be able to evaluate if there is a correlation between these hematological variables and the results of these tests.

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All authors declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964, as revised in 2013. Informed consent was obtained from all patients for being included in the study.

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ORIGINAL ARTICLE

The Chronic Oral Administration of Clobenzorex or Amphetamine Decreases Motor Behavior and Induces Glial Activation in the Striatum Without Dopaminergic Degeneration

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Abstract

Described as amphetamine-like due to their structural and stimulant similarities, clobenzorex is one of the five most-commonly used drugs in Mexico for the treatment of obesity. Various studies have shown that amphetamines induce dopaminergic neurotoxicity and neuroinflammation in the striatum, symptoms which are associated with motor damage. For this reason, the present study aimed to evaluate the effect of chronic clobenzorex administration on motor behaviors, TH immunoreactivity, gliosis, and the neurodegenerative process in the striatum and substantia nigra pars compacta (SNpc). The present research was conducted on three experimental groups of male Wistar rats: the vehicle group, the amphetamine group (2 mg/kg), and the clobenzorex group (30 mg/kg). All groups were subject to oral administration every 24 h for 31 days. Motor activity and motor coordination were evaluated in the open field test and the beam walking test, respectively. The animals were euthanized after the last day of treatment to enable the extraction of their brains for the evaluation of tyrosine hydroxylase (TH) levels, the immunoreactivity of the glial cells, and the neurodegeneration of both the striatum and SNpc via amino-cupric-silver stain. The results obtained show that amphetamine and clobenzorex administration decrease motor activity and motor coordination in the beam walking test and cause increased gliosis in the striatum, while no significant changes were observed in terms of immunoreactivity to TH and neurodegeneration in both the striatum and SNpc. These results suggest that the chronic administration of clobenzorex may decrease motor function in a manner similar to amphetamine, via the neuroadaptive and non-neurotoxic changes caused to the striatum under this administration scheme.

Keywords Amphetamine · Clobenzorex · Motor behaviors · Striatum · Gliosis · Neurodegeneration

Introduction

Clobenzorex hydrochlorate, trade name Asenlix, is an N-substituted compound of S (+) - amphetamine. In Mexico, it is one of the five main anorectic drugs used to treat obesity (Espinoza-Franco and Morín-Zaragoza 2013). Although the US Food and Drug Administration withdrew clobenzorex from the market some years ago, there is evidence of its availability along the USA-Mexico border (Tong et al. 2002). A controlled drug sold only by prescription, clobenzorex is available on the illicit market, where it is sold as a recreational drug (Cornaert et al. 1986; Baumevieille et al. 1997). Athletes have been reported to use it to improve their performance (Docherty 2008), while, in Brazil, it has been identified in pills seized from truck drivers who consume them to remain awake and reduce fatigue when driving long distances (Moreira et al. 2016).

Clobenzorex and amphetamine derivatives are metabolized into amphetamine and excreted in the urine (Glasson et al. 1971; Valtier and Cody 2000). Some studies have shown that the consumption of clobenzorex produces hepatotoxicity (Tong et al. 2002) and cardiovascular side effects such as heart failure, arterial hypertension, and pulmonary artery

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hypertension (Cornaert et al. 1986; Seferian et al. 2013; García-Alonso et al. 2019). However, its toxic effects on the central nervous system are unknown. Young et al. showed that clobenzorex exerts stimulating effects on the central nervous system in a manner similar to that exerted by amphetamines (Young et al. 1997). Furthermore, it has been shown that, after oral administration, up to 15% of the initial clobenzorex dose is metabolized to d-amphetamine (Valtier and Cody 2000), which could contribute to the prolongation of its stimulating effects.

Amphetamines and their derivatives (methamphetamine, or METH, and methylenedioximemethamphetamine, or MDMA) are neurotoxic psychostimulant drugs that induce short- and long-term effects. In the short term, amphetamines cause euphoria, increased heart rate, hypertension, hyperthermia, excitement, alertness, and wakefulness (Homer et al. 2008; Moratalla et al. 2017). The chronic administration of amphetamine in laboratory animals leads to dopaminergic neurotoxicity, a finding which has generated considerable concern about the use of these compounds both in clinical practice and recreationally (Eisch et al. 1992). The alteration of the function of the vesicular monoamine transporter (VMAT) and the dopamine reuptake protein (DAT) promote increased dopamine (DA) levels (Sulzer et al. 2005; German et al. 2015). Furthermore, amphetamines can increase serotonergic (5HT) and noradrenergic neurotransmission (Heal et al. 2013), while the long-term use of amphetamines has been associated with cognitive dysfunction and motor deficits in both humans and animal models (Ornstein et al. 2000; Volkow et al. 2001; Moratalla et al. 2017). Amphetamine-induced motor behavior deficits are mainly related to neurotoxicity in the nigrostriatal dopamine system (Walsh and Wagner 1992; Ares-Santos et al. 2014). This dopaminergic neurotoxicity is characterized by a decrease in DA and its metabolites and a decrease in the levels of the enzyme tyrosine hydroxylase (TH) and the dopamine transporter in the striatum (Quinton and Yamamoto 2006). Acutely high amphetamine doses have been shown to induce neurodegeneration in the striatum and SNpc (Ricaurte et al. 1982; O'Callaghan et al. 1995; Ares-Santos et al. 2014).

Studies conducted on the neurotoxicity mechanism of amphetamines have involved oxidative stress, excitotoxicity, mitochondrial dysfunction, energy failure, and neuroinflammation (Cadet et al. 2007; Yamamoto et al. 2010; Halpin et al. 2014). Most of said studies show damage to the dopaminergic nerve terminals in METH or MDMA binge-dosing models (Krasnova and Cadet 2009; Moratalla et al. 2017). Various studies have shown that amphetamine exposure induces neuroinflammation (Krasnova and Cadet 2009; Northrop and Yamamoto 2014), glial reactivity (which includes the release of cytokines, chemokines, reactive oxygen, and nitrogen species), and blood-brain barrier dysfunction (Kousik et al. 2012; Clark et al. 2013). The activation of microglia and astrocytes is a well-characterized response to amphetamines that induce damage to the monoamine nerve terminals of the striatum (O'Callaghan et al. 1995; Thomas et al. 2004; Friend and Keefe 2013). To date, the long-term effects of clobenzorex on cellular mechanisms and motor behavior are unknown, despite it being an amphetamine-type drug and subject to illicit use. The present study aimed to evaluate the effects of the chronic administration of clobenzorex on motor behaviors, TH immunoreactivity, glial cell reactivity, and the neurodegenerative process in the striatum and SNpc.

Materials and Methods

Animals

Adult male Wistar rats (350–450 g) ($n = 42$) were obtained from the *Claude Bernard* animal facilities at the *Benemérita Universidad Autónoma de Puebla* (BUAP). The animals were individually housed in groups of three–four per cage under constant temperature conditions (22 ± 2 °C) and a 12-h light/dark cycle (lights on at 08.00), with food and water given ad libitum. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals set out in the corresponding Official Mexican Standard (NOM-062-ZOO-1999) and were approved by the BUAP bioethics committee.

Drug Treatments

(+) Amphetamine hydrochloride (Sigma-Aldrich Ltd., St. Louis, MO, USA) and clobenzorex hydrochloride were dissolved in isotonic saline solution (ISS) to obtain final concentrations of 1 mg/mL and 9 mg/mL, respectively. The amphetamine and clobenzorex doses were 2 mg/kg and 30 mg/kg, respectively. The clobenzorex dose was selected in accordance with that reported by Young et al. (1997), who found that clobenzorex is 20 times less potent than amphetamine. The amphetamine dose was selected based on that reported by Armstrong et al. (2004), who observed that it was the minimum repeated amphetamine dose for which an astrocytosis response has been observed. The pharmacological treatments prepared shortly before daily oral administration for 31 days from 14:00 to 16:00 pm (Fig. 1).

Rectal Temperature

The rectal temperature of the animals was monitored using a digital thermometer (Citizen CTA301) lubricated with Vaseline and inserted into the rectum of the animals up to a depth of 2 cm. The basal temperature was recorded prior to the administration of the drugs and every 2 h post-administration.

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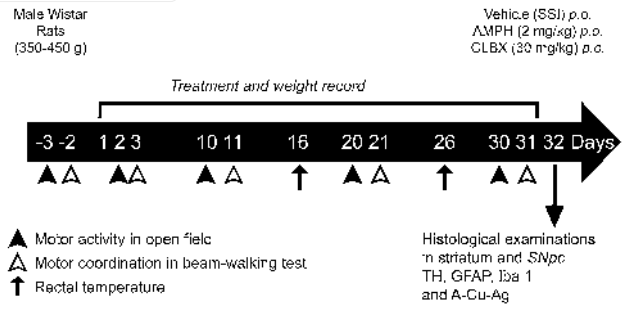

Fig. 1 Scheme of the experimental sequence. Three groups were obtained: vehicle (SSI); clobenzorex (30 mg/kg); and amphetamine (2 mg/kg). Every 10 days, the animals were tested for motor activity and motor coordination prior to the administration of the drugs. Finally, the histological examinations were performed in the striatum and SNpc

Motor Behaviors

The time sequences for the behavioral tests are shown in Fig. 1. The motor behaviors presented by the animals were evaluated before the daily administration of each drug, in order to assess the consequences of the previous doses administered on motor function. Motor activity in the open field test was evaluated on days 2, 10, 20, and 30 post-treatment, while the narrow beam walking test was conducted on days 3, 11, 21, and 31 post-treatment. All the behaviors were carried out at 08:00–12:00 h and a temperature of 22 ± 2 °C, in a sound-insulated room.

Motor Activity in the Open Field Test

The animals were subject to a motor activity test four times over the course of the open field model experiment (Opto-Varimex 5, Columbus Instruments, OH, USA). This test evaluates the distance travelled (cm), the average speed (cm/s), and the number of rearings made over a 15-min period. The present study used transparent acrylic boxes, 44 cm long by 40 cm wide and 20 cm high, for this test, with the data generated then captured, recorded, and analyzed in the software Auto-Track.

Assessment of Motor Balance and Coordination in the Narrow Beam Walking Test

The motor coordination of the animals was evaluated in the narrow beam walking test in accordance with the protocol previously reported by Patricio et al. (2019). The narrow beam consisted of a wooden beam, 120 cm long, 2.5 cm wide, and 2.5 cm high and suspended 43 cm off the ground on wooden supports. A dark box, 15 cm × 30 cm × 20 cm was placed at the end of the beam. Each animal was trained to use the beam for one day four days prior to the administration of each drug, undergoing five trials, with a 1-h interval between each trial, until it could cross the beam and reach the dark box. If the rat stopped on the beam to look around, the researcher pushed it gently from behind with their fingers. Once the animals had been trained on the beam, the administration of each of the drugs of interest began on day 1, with motor coordination analyzed again on days 3, 11, 21, and 31 of administration. Evaluating the latency to cross the beam and the number of errors, each test comprised three trials with a maximum duration of 90 s, with a 1-h interval applied between trials. An error was defined as any incident of slipping on the surface of the beam, loss of balance, or a fall from the beam (Lezcano 2009; Biernaskie 2004), with a fall recorded as the maximum time permitted. The task was considered as completed when the rat had completely placed all four limbs in the dark box at the opposite end of the beam.

Histological Examinations

One day after the final administration of each drug, all animals ($n = 14$ per group) were anesthetized and intracardially perfused with 200–250 mL 4% paraformaldehyde in borate buffer (pH = 7.4). The brains were removed and stored in a 30% sucrose solution, after which 50-µm coronal sections of the striatum and the SNpc were obtained in a vibratome (Leica VT1000S). The sections intended for A-Cu-Ag staining were kept in 4% paraformaldehyde in borate buffer, while the sections to be used for the immunohistochemical analysis for TH, GFAP, and Iba-1 were kept in phosphate-buffered saline (PBS, pH 7.4). The striatum sections were taken, approximately, at 1.7 to −0.4 mm, while the SNpc sections were taken, approximately, −5.2 to −6.0 mm from the bregma.

Immunohistochemistry Analysis for Tyrosine Hydroxylase

The coronal sections taken from each subject were washed three times in 0.2% PBS-Triton X-100 for 10 min in order to impregnate the tissue. The endogenous peroxidase was deactivated via H_2O_2 (3%) and methanol (10%) incubation for 20 min, with the tissue samples then washed three times with PBS-Triton for 10 min each wash. The non-specific binding sites were blocked by means of incubation in IgG-free 2% bovine serum albumin (BSA, Sigma) for 60 min, with the sections then incubated for two nights at 4 °C with the monoclonal mouse antibody anti-tyrosine hydroxylase (TH) (Merck Millipore, USA), diluted to 1:1000. After 48 h of incubation with the primary antibody at 4 °C, the brain tissue sections were incubated in biotinylated mouse secondary antibody (Vector Laboratories, Burlingame, CA) and exposed to an avidin-biotin

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complex solution (Thermo Fisher Scientific). Finally, the tissue sections were incubated with 0.05% diaminobenzidine and 0.03% hydrogen peroxide and then mounted on gelatin-coated slides, dehydrated in an ascending series of alcohols, cleared in xylene, and coverslipped using PolyMount (PolyScience, USA).

The images of the striatum and SNpc were observed using a Leica DM/1000 microscope (Leica DM/1000 Microsystems) at 1.5× and 10×, respectively, while the photographs were taken using a Jenoptik ProgRes C14 plus digital camera and ProgRes software.

The TH levels in the striatum were quantified using the ImageJ program to graph the percentage of the area that had been stained. The number of TH-positive neurons in the SNpc was established using an image analysis tool based on the ImageJ program. The data are represented as the number of TH immunoreactive neurons in the SNpc.

Immunohistochemistry for GFAP and Iba-1

Immunoreactivity to glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (Iba1) were used to identify astrocytes and microglia cells, respectively. The presence of GFAP and Iba1 was determined by means of the use of specific antibodies in free-floating brain sections, which were permeated with 0.2% Triton X-100 in PBS for three wash cycles, each comprising three 10-min washes. The non-specific binding sites were blocked via incubation in IgG-free 2% bovine serum albumin (BSA, Sigma) for 60 min. The coronal brain sections were incubated overnight at 4 °C in the corresponding antibody—either polyclonal anti-GFAP rabbit antibody, diluted to 1/500 (Dako, Denmark A/S), or rabbit anti-Iba1 monoclonal antibody, diluted to 1/500 (Wako Pure Chemical Industries, Ltd. Osaka, Japan). Primary antibody labeling was undertaken using a secondary conjugated rhodamine (GFAP) and visualized in the red channel, while the primary antibody was recognized using a secondary conjugated FICT (Iba1) and visualized in the green channel. The sections were mounted on gelatin-subbed glass slides and covered with Mowiol-DABCO resin (Sigma-Aldrich). The immunostained cells in the dorsomedial and dorsolateral striatum were observed through a Leica DM/1000 microscope (Leica DM/1000 Microsystems) at 20×, while the photographs were taken using a Jenoptik ProgRes C14 plus digital camera and the ProgRes software.

The digitized images were converted into Tagged Image File Format files to facilitate subsequent analysis. For GFAP and Iba1, solely the total area of the photomicrograph that presented fluorescent staining was analyzed, with the threshold then chosen from the vehicle group and applied to all experimental groups.

Amino-cupric-silver (A-Cu-Ag) Stain

Neuronal degeneration was analyzed by means of A-Cu-Ag staining, applied in accordance with the protocol previously reported by Ares-Santos et al. (2014), which specifically stains the dendritic trees and cell bodies of damaged neurons and axons during the early or later stages of neurodegeneration (de Olmos et al. 1994; Switzer 2000). The immunostained cells of the striatum were observed through a Leica DM/1000 microscope at 20× and those of the SNpc at 1.5×. The percentage of the area that was stained was calculated using the ImageJ image analysis program to convert color intensities to grayscale, with the threshold chosen for the vehicle group.

Statistical Analysis

The data were analyzed by means of a Shapiro-Wilk normality test, with the data presenting parametric behavior analyzed using a one-way or two-way ANOVA and presented here as mean ± standard error of the mean (SEM). Relevant differences were analyzed pairwise via a Student's *t*-test or a post hoc Tukey test. The data presenting non-parametric behavior were analyzed using a Kruskal-Wallis test and Dunn's multiple comparisons test. The criterion for significance was $p < 0.05$.

Results

The Chronic Oral Administration of Clobenzorex or Amphetamine Decreases Body Weight and Increases Rectal Temperature

Clobenzorex or amphetamine treatment was administered for 31 days, with the results obtained showing that both treatments decreased the body weight of treated animals significantly more than that of the control group ($p < 0.05$) (Fig. 2a).

The rectal temperature of the subjects was measured at 0, 60, and 120 min on the 13th and 26th days post-treatment. On day 13, the rats administered with amphetamine did not show significant changes in rectal temperature; however, those administered with clobenzorex showed a significant increase in temperature from minute 60 onwards (mean 38.0), which remained until minute 120 (mean 38.11) and was higher than that recorded for the control group (mean 37.2; 37.1) (Fig. 2b). By day 26, it was found that both the amphetamine and clobenzorex groups presented a significantly higher rectal temperature, at minute 60 (mean 37.4; 37.8), than that found for the vehicle group (mean 36.8). At minute 120, only the clobenzorex group presented a higher temperature than the vehicle group (Fig. 2c).

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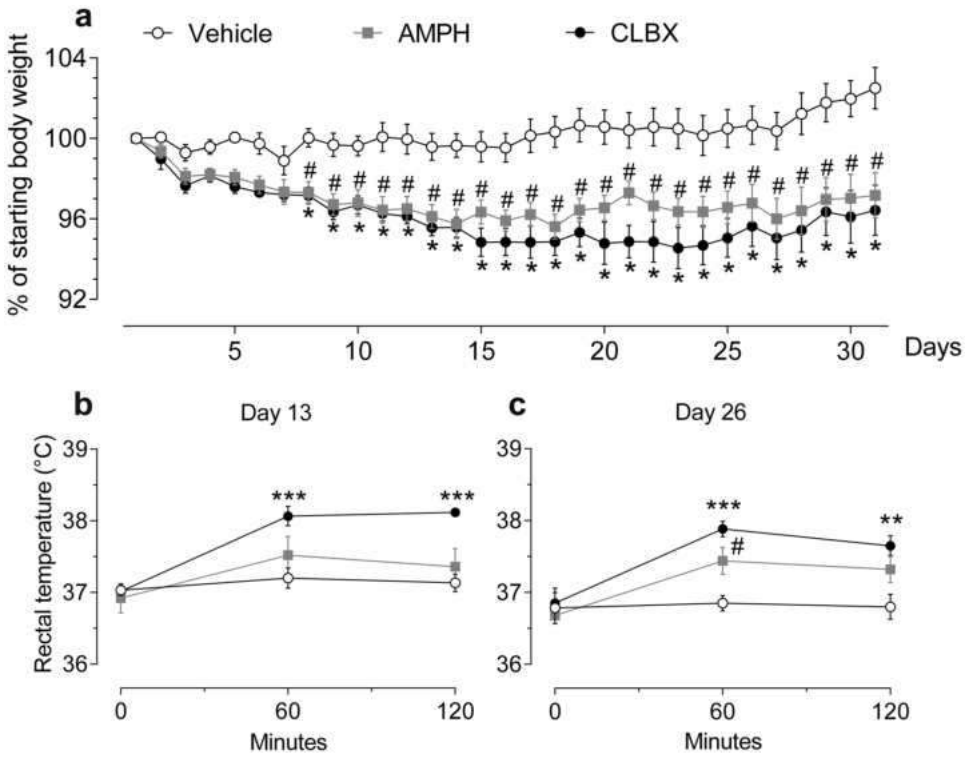

Fig. 2 Chronic administration of clobenzorex or amphetamine decreases body weight and modifies rectal temperature. (**a**) Clobenzorex and amphetamine decrease body weight. Data represent mean ± SEM, $n = 14$ per group. (**b, c**) Clobenzorex induces increased body temperature on days 13 and 26 of treatment. Data represent mean ± SEM. Statistical analysis was conducted via two-way ANOVA for repeated measures and a Tukey post-test. *$p < 0.05$ clobenzorex vs vehicle; **$p < 0.01$ clobenzorex vs vehicle; ***$p < 0.001$ clobenzorex vs vehicle; #$p < 0.05$ amphetamine vs vehicle

Similar to Amphetamine, Oral Clobenzorex Administration Decreases Locomotor Activity

Motor activity was evaluated in the open field test on days 2, 10, 20, and 30 post-treatment prior to the daily administration of clobenzorex or amphetamine. The results obtained show that clobenzorex or amphetamine treatment decreases horizontal and vertical locomotor activity from the tenth day post-treatment onwards. The distance traveled in meters on day 2 did not change for the vehicle (mean 41.0), amphetamine (mean 42.4), and clobenzorex (mean 45.7) groups. The amphetamine treatment was found to achieve a significantly lower distance traveled in the open field model, on days 10 (mean 27.3; $p < 0.001$), 20 (mean 30.9; $p < 0.001$), and 30 (mean 26.1; $p < 0.001$), than that observed for the vehicle group (mean 44.9; 46.4; and 44.6). Similarly, treatment with clobenzorex was found to achieve a significantly lower distance traveled, by days 20 (mean 38.9; $p < 0.001$) and 30 (31.1; $p < 0.01$), than that observed for the vehicle group (Fig. 3a). The average speed (cm/s) observed on day 2 did not change for the vehicle (mean 5.6), amphetamine (mean 6.0), and clobenzorex (mean 6.1) groups. The amphetamine treatment was observed to have reduced the average speed by days 10 (mean 4.7; $p < 0.001$), 20 (mean 4.8; $p < 0.001$), and 30 (mean 4.4; $p < 0.001$) significantly more than that observed for the vehicle group (mean 6.1; 6.2; and 5.9). The clobenzorex treatment had decreased the average speed by days 10 (mean 5.2; $p < 0.05$), 20 (mean 4.5; $p < 0.001$), and 30 (4.6; $p < 0.01$) significantly more than that observed for the vehicle group (Fig. 3b). The number of rearings recorded on day 2 did not change for the vehicle (mean 58.7), amphetamine (mean 48.2), and clobenzorex (mean 54.6) groups. The amphetamine treatment had reduced the number of rearings by days 10 (mean 27.4; $p < 0.01$), 20 (mean 35.4; $p < 0.05$), and 30 (mean 33.6; $p < 0.05$) significantly more than that observed for the vehicle group (mean 51.1; 52.2; and 54.3). Similarly, the clobenzorex treatment had decreased the number of rearings by days 20 (mean 29.8; $p < 0.01$) and 30 (33.6; $p < 0.05$) significantly more than that observed for the vehicle group (Fig. 3c). The findings of the present study show that clobenzorex induces a deficit in locomotor activity in a similar way to amphetamine.

Similar to Amphetamine, the Oral Administration of Clobenzorex Impairs Motor Coordination

Motor coordination was evaluated in the beam walking test on days 3, 11, 21, and 31 post-treatment, prior to the daily administration of clobenzorex or amphetamine. The time taken by each of the experimental groups to cross the beam was quantified, although no significant differences were found for the vehicle, clobenzorex, and amphetamine groups (Fig. 3d). On the other hand, in terms of the number of errors made by the animals, the amphetamine and clobenzorex groups had not shown significant changes by day 3. Treatment with amphetamine was found to have increased the

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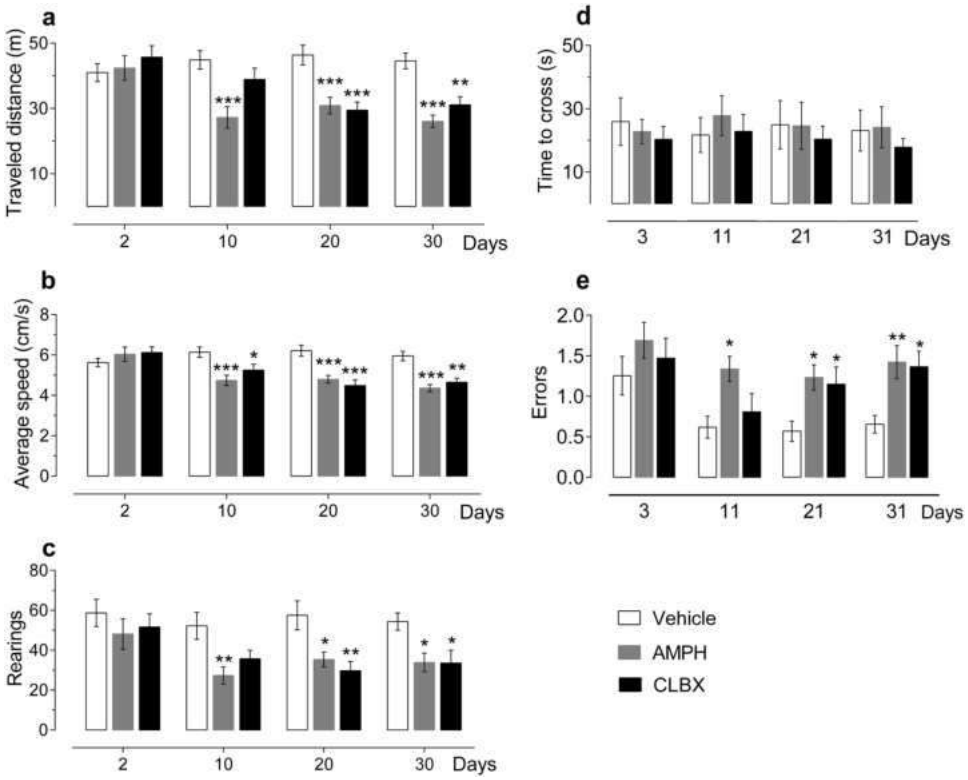

Fig. 3 Chronic clobenzorex or amphetamine administration decreases locomotor activity in the open field test and motor coordination. Motor activity was evaluated by (**a**) distance travelled, (**b**) average speed, and (**c**) number of rearings. (**d**) Time to cross beam and (**e**) number of errors in the narrow beam test. Data show mean ± SEM, $n = 14$ per group. Statistical analysis was conducted via two-way ANOVA for repeated measures and a Tukey post-test. *$p < 0.05$ vs vehicle; **$p < 0.01$ vs vehicle; and ***$p < 0.001$ vs vehicle

number of errors by days 11 (mean 1.3, $p < 0.05$), 21 (mean 1.2, $p < 0.05$), and 31 (mean 1.4; $p < 0.01$) significantly more than that observed for the vehicle group (mean 0.6, 0.6, and 0.7). The group administered with clobenzorex had shown an increase, in the number of errors by days 21 (mean 1.1, $p < 0.05$) and 31 (mean 1.4; $p < 0.05$) post-administration, that was higher than that observed for the vehicle group (Fig. 3e). The results indicate that motor behavior is affected by clobenzorex and amphetamine treatment.

Chronic Oral Administration of Clobenzorex or Amphetamine Modifies Neither Tyrosine Hydroxylase Levels Nor Neurodegeneration

The present study aimed to ascertain whether the motor effects induced by clobenzorex or amphetamine were related to damage sustained to the dopaminergic system. To this end, on day 31, the percentage area of the striatum that was stained and, thus, the level of TH were established, while both the number of TH-positive cells in the SNpc and the level of neurodegeneration were evaluated via A-Cu-Ag staining (Figs. 4 and 5). The results obtained show that treatment with either amphetamine (mean 83.1) or clobenzorex (mean 83.0) does not modify the percentage area stained for the TH enzyme in the striatum to a greater degree than that observed for the control group (mean 82.5) (Fig. 4d). The evaluation of the SNpc revealed that treatment with amphetamine (mean 171.3) or clobenzorex (mean 154.0) did not modify the number of +TH cells to a greater degree than that observed for the control group (mean 153.2) (Fig. 5d). Furthermore, treatment with amphetamine (mean 0.041, 0.34) or clobenzorex (mean 0.12, 0.35) did not increase neurodegeneration in the striatum and SNpc significantly more than the control group (mean 0.05, 0.50) (Figs. 4e and 5e).

The Chronic Oral Administration of Clobenzorex or Amphetamine Increases Gliosis in the Striatum

Immunohistochemical staining via GFAP and Iba-1 was applied to determine astrocyte and microglial activation (Figs. 6 and 7). The results obtained showed that 31 days after the oral administration of clobenzorex, the levels of GFAP and Iba-1 in the dorsomedial striatum (DMS) increased by 11.8% and 3.4%, respectively, while the vehicle group presented a GFAP-stained area of 1.7% and an Iba-1-stained area of 1.7% ($p < 0.001$). The amphetamine group showed larger percentage areas of GFAP and Iba-1 staining in the DMS, 4.4% and 2.9%, respectively, than those observed for the vehicle group ($p < 0.05$). Moreover, it was observed that the clobenzorex group presented a larger percentage area stained by GFAP than that observed for the amphetamine group ($p < 0.01$) (Fig. 6).

The immunoreactivity of GFAP and Iba-1 in the dorsolateral striatum (DLS) was also analyzed (Fig. 7). The results obtained found that the oral administration of clobenzorex caused a significantly higher percentage of the area of the

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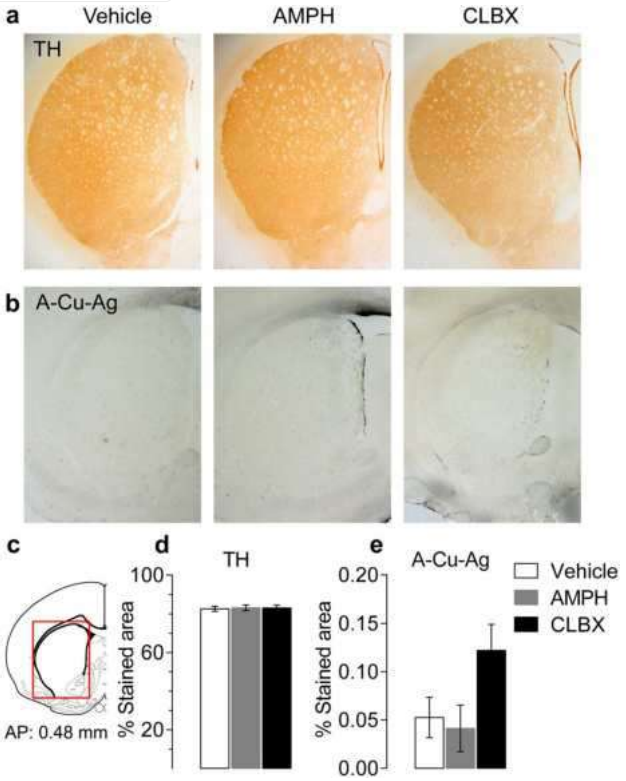

Fig. 4 Chronic clobenzorex or amphetamine administration does not modify TH expression and degeneration in the striatum. Photomicrographs show (**a**) the immunoreactivity of TH, (**b**) amino-cupric-silver (A-Cu-Ag) staining, and (**c**) scheme of striatum localization in a rat brain coronal section. (**d**) Histograms show the percentage of the total area stained for TH in the striatum. The values show mean ± SEM, $n = 6$ per group. The statistical analysis was conducted via one-way ANOVA and a post hoc Tukey test. (**e**) Histograms show the percentage of A-Cu-Ag staining in the striatum. The values show mean ± SEM, $n = 4$ per group. Statistical analysis was conducted via a Kruskal-Wallis test and Dunn's multiple comparisons

DLS that was stained, 6.9% and 3.9% for GFAP and Iba-1 ($p < 0.001$, $p < 0.01$), respectively, than that observed for the vehicle group (1.7% for the GFAP-stained area and 2.1% for the Iba-1-stained area). The amphetamine group showed greater percentages of GFAP and Iba-1-stained areas in the DLS, 3.9% and 3.3%, respectively, than those observed for the vehicle group ($p < 0.05$). The findings of the present study show that the chronic administration of clobenzorex or amphetamine increased GFAP and Iba-1 levels in both the DMS and DLS.

Discussion

Clobenzorex is a drug used for both therapeutic purposes and recreationally (Cornaert et al. 1986; Baumevieille et al. 1997; Tong et al. 2002; Docherty 2008; Espinoza-Franco and Morín-Zaragoza, 2013; Moreira et al. 2016). Therefore, there is a critical need to understand the cellular mechanisms involved in triggering both toxic effects in the brain and motor behavior. The present study found that chronic treatment with clobenzorex or amphetamine causes a decrease in locomotor activity and an alteration in motor coordination. The alterations in motor function were accompanied by the activation of glial cells in the dorsal striatum, with no evidence found of nigrostriatal dopaminergic neurotoxicity after chronic exposure. In addition, a decrease in body weight and an increase in body temperature were observed.

The results obtained indicate that treatment with clobenzorex or amphetamine reduces the subject's body weight and coincide with the findings of other research groups that show that the chronic administration of METH at low doses causes a decrease in body weight (Lloyd et al. 2017; Thanos et al. 2016). It has been proposed that the decrease in body weight observed with the administration of amphetamine is attributed to the decreased food intake caused by the appetite suppression associated with this drug (Shu-Chen et al. 2014). This suggests that the doses of amphetamine and clobenzorex used in the present study have anorexic effects. The appetite suppression induced by amphetamines (including clobenzorex) is mediated by increased levels of monoamines in the paraventricular nucleus of the hypothalamus, while the activation of the noradrenergic (Wellman et al. 1993) and serotonergic (Bovetto and Richard 1995) systems is involved in suppressing food intake. Acute exposure to amphetamines has been shown to cause weight loss, which has been associated with changes in peripheral hormones such as ghrelin, leptin, and the neuropeptide Y (Kobeissy et al. 2008a, b). Both peripheral and central mechanisms could be used to mediate the decrease in body weight induced by the chronic administration of amphetamine or clobenzorex, as evidenced in the present study.

Additionally, the daily intraperitoneal administration of METH at 4 and 8 mg/kg in rats for 16 weeks has been shown to decrease both body weight and food intake (Thanos et al. 2017) due to changes in the peripheral signals that control satiety. A decrease in food intake has been found with the use of METH in humans and rodents and is related to a modification in leptin, ghrelin, and neuropeptide Y signaling (Comer et al. 2001; Kirkpatrick et al. 2019; Kobeissy et al. 2008a, b). This finding rules out the conclusion that the reduction in locomotor activity observed in the present study is due to reduced food consumption, as the neuronal circuits involved are different.

On days 13 and 26 of the present study, the administration of clobenzorex caused an increase in body temperature 120 min after administration. The increase in body temperature induced by clobenzorex could be related to the activation of the neuroendocrine axis, as well as the release of monoamines at a central and peripheral level, an effect also induced

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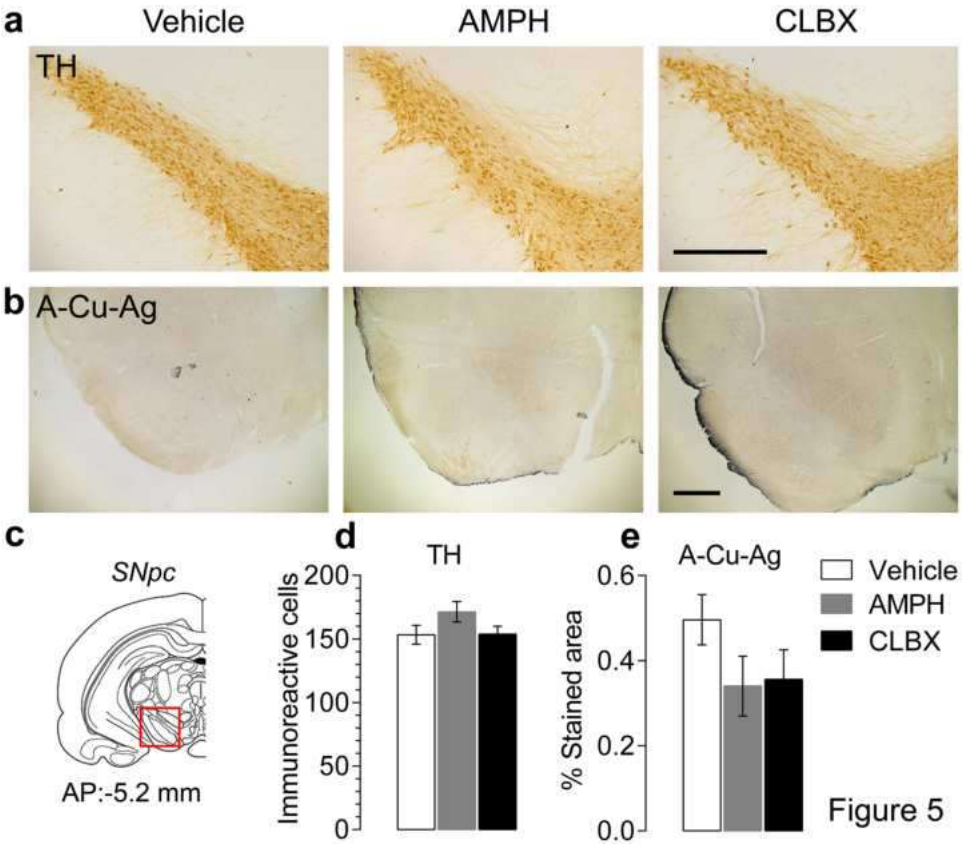

Fig. 5 Chronic clobenzorex or amphetamine administration does not modify the expression of TH or degeneration in the SNpc. Photomicrographs show the immunoreactivity of TH (**a**) and amino-cupric-silver (A-Cu-Ag) staining (**b**). (**c**) Scheme of SNpc localization in a rat brain coronal section. (**d**) Histograms show the number of TH immunoreactive cells in the SNpc. The values show mean ± SEM, $n = 6$ per group. Statistical analysis was conducted via one-way ANOVA and a post hoc Tukey test. (**e**) Histograms show the percentage area of A-Cu-Ag staining in the SNpc. The values show mean ± SEM, $n = 4$ per group. Statistical analysis was conducted via a Kruskal-Wallis test and Dunn's multiple comparisons. Bar indicates 500 µm

by amphetamine derivatives (Matsumoto et al. 2014). Thermogenesis induced via METH or MDMA has been shown to promote norepinephrine release in the sympathetic nervous system, the activation of the hypothalamic-pituitary-thyroid axis, and glucocorticoid secretion in the adrenal cortex (Makisumi et al. 1998; Sprague et al. al. 2003). Clobenzorex treatment in humans has not been associated with hyperthermia at therapeutic doses (Espinoza-Franco and Morín-Zaragoza 2013). Our results show that the administration of clobenzorex significantly increases rectal temperature without reaching hyperthermia, with, on day 26, the amphetamine group presenting an increase in rectal temperature 60 min post-administration, which is due to the low dose (2 mg/kg) administered in the present study. In binge-dosing models, the lowest dose of amphetamine or METH capable of producing hyperthermia is 3–4 mg/kg (Levi et al. 2012; McConnell et al. 2015).

It is well-described that locomotor activity is stimulated by the acute administration of amphetamines (Hirabayashi et al. 1981; Young et al. 1997; Heal et al. 2013), including clobenzorex (Young et al. 1997). The persistent administration of amphetamine stimulants has been shown to damage motor behavior in animal models (Walsh and Wagner 1992; Wallace et al. 2001; Timár et al. 2003; Ares-Santos et al. 2014; Moratalla et al. 2015). However, the foregoing studies evaluate motor impairment after the acute administration of high doses of METH or MDMA. For the first time, the present study shows that prolonged treatment with clobenzorex has detrimental effects on locomotor activity. The chronic administration of amphetamine shows a decrease in locomotor activity that occurs even earlier than that observed for the clobenzorex group. This disparity indicates that clobenzorex takes longer than amphetamine to produce the same impairment in motor function. Furthermore, treatment with clobenzorex and amphetamine resulted in a significant increase in the number of errors observed in the beam walking test, evidencing a decrease in fine motor coordination. These results suggest that chronic treatment with clobenzorex results in impaired motor behavior similar to that observed with amphetamine treatment. Impaired motor function, specifically the impairment of both gross motor function performance and fine motor coordination, has been reported in chronic METH abusers (Volkow et al. 2001; Kalechstein et al. 2003). These findings could explain the risk of fatal traffic accidents for truck drivers who use stimulants to keep themselves awake (Logan 1996; Crifasi and Long 1996; Gates et al. 2013). Epidemiological studies conducted between 1998 and 2015 have found that amphetamines are one of the main drug types associated with traffic accidents (Gjerde et al. 2015). Furthermore, a positive relationship between blood amphetamine concentrations and impaired driving ability has been demonstrated (Gustavsen et al. 2006). Thus, the motor deficits induced by the administration of

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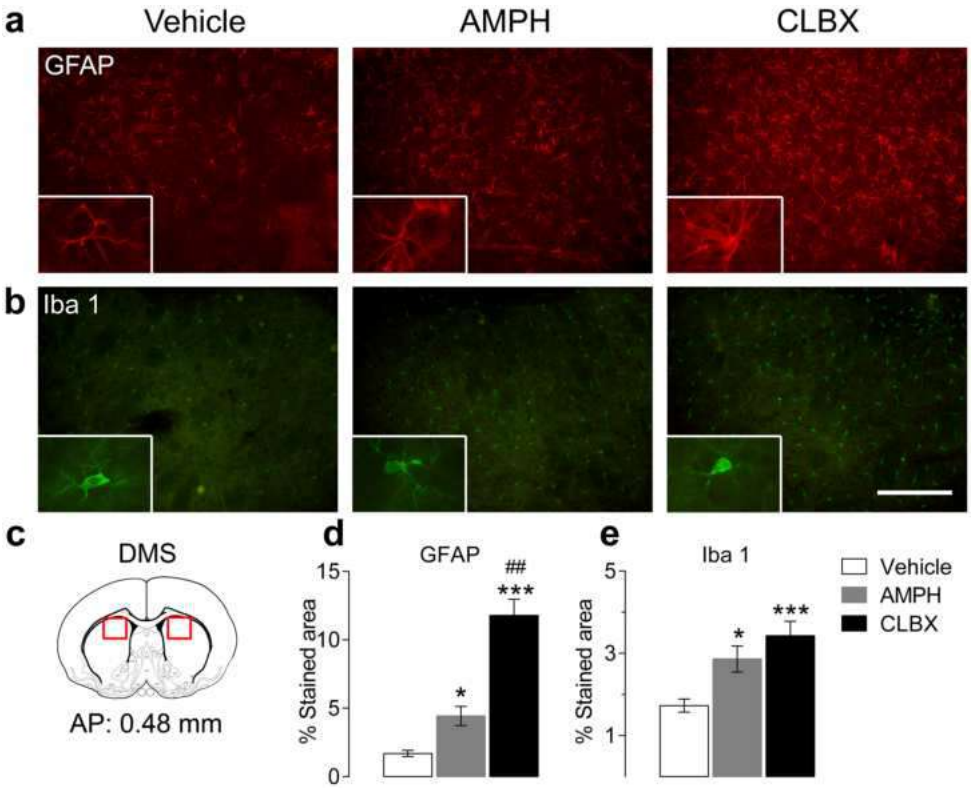

Fig. 6 Chronic clobenzorex or amphetamine administration induces expression of GFAP and Iba-1 in the dorsomedial striatum. Photomicrographs of the dorsomedial striatum (DMS) section, stained with GFAP (**a**) and Iba-1 (**b**). (**c**) Scheme of the DMS in a rat brain coronal section. Histograms show the proportional GFAP-ir (**d**) and Iba-1-ir (**e**) stained areas. The values show ± SEM, $n = 4$ per group. Statistical analysis was conducted with a Kruskal-Wallis analysis and Dunn's multiple comparisons test. *$p < 0.05$ vs vehicle; ***$p < 0.001$ vs vehicle; ##$p < 0.01$ vs amphetamine. Bar indicates 200 µm

clobenzorex could have an impact on the motor behavior of those truck drivers who consume it.

Amphetamine-induced motor disturbances mainly occur due to dopaminergic neurotoxicity in the dorsal striatum in binge-dosing models (Walsh and Wagner 1992; Moratalla et al. 2017). However, the present study shows that the 31-day administration of amphetamine or clobenzorex does not result in the loss of dopaminergic terminals in either the striatum or the respective cell bodies of the SNpc. The evaluation of neurodegeneration via A-Cu-Ag staining conducted in the present research revealed no neurodegeneration in either brain region. These results suggest that the decreased motor function caused by the chronic administration of clobenzorex is not related to a decrease in nigrostriatal dopaminergic innervation. Le Cozannet et al. (2013) showed that the intravenous self-administration of METH in rats induces hypolocomotion without presenting dopaminergic decrease in the striatum. However, other studies have shown alterations in striatal dopaminergic neurotransmission after prolonged exposure to METH (Segal et al. 2005; Lacán et al. 2013; Thanos et al. 2016), without the loss of SNpc cells (Lacán et al. 2013). This suggests that neuroadaptive changes occur in the striatal dopaminergic system, in response to prolonged exposure to clobenzorex and amphetamine that result in decreased locomotor activity and motor coordination.

On the other hand, a single dose of amphetamine (10 mg/kg) has been shown to damage the dopaminergic system via mechanisms such as excitotoxicity, oxidative stress, and energy failure (mitochondrial dysfunction) (Tung et al. 2017). Another study, which measured striatal DAT, TH, and DA levels, found that the acute administration of four 5 mg/kg amphetamine injections at 2-h intervals decreases the integrity of the dopaminergic terminals (Angoa-Perez et al. 2013). However, despite the significant evidence of the neurotoxicity induced by amphetamine, there is little evidence demonstrating its effect on neurodegeneration. Ricaurte et al. (1984) showed that the continuous administration of amphetamine or methamphetamine, at a dose of 4 mg/kg for 3 days, increases the amount of fine granular argyrophilic debris indicative of the degeneration of striatal fibers. However, it has been shown that a single injection of a high dose of amphetamine, 30 mg/kg, does not induce neuronal death in the rat striatum, as determined 72 h after treatment using the Fluoro-Jade C stain (Marques et al. 2008). The present study found no evidence of neurodegeneration in either the striatum or the SNpc after 31 days of clobenzorex or amphetamine treatment. It should be noted that Ricaurte et al. did find evidence both of the induction of the neurodegenerative process via the administration of amphetamine at a dose higher than that used in the present study and of the short-term toxic effects of amphetamine.

One possible mechanism caused by chronic exposure to clobenzorex or amphetamine is the downregulation and/or

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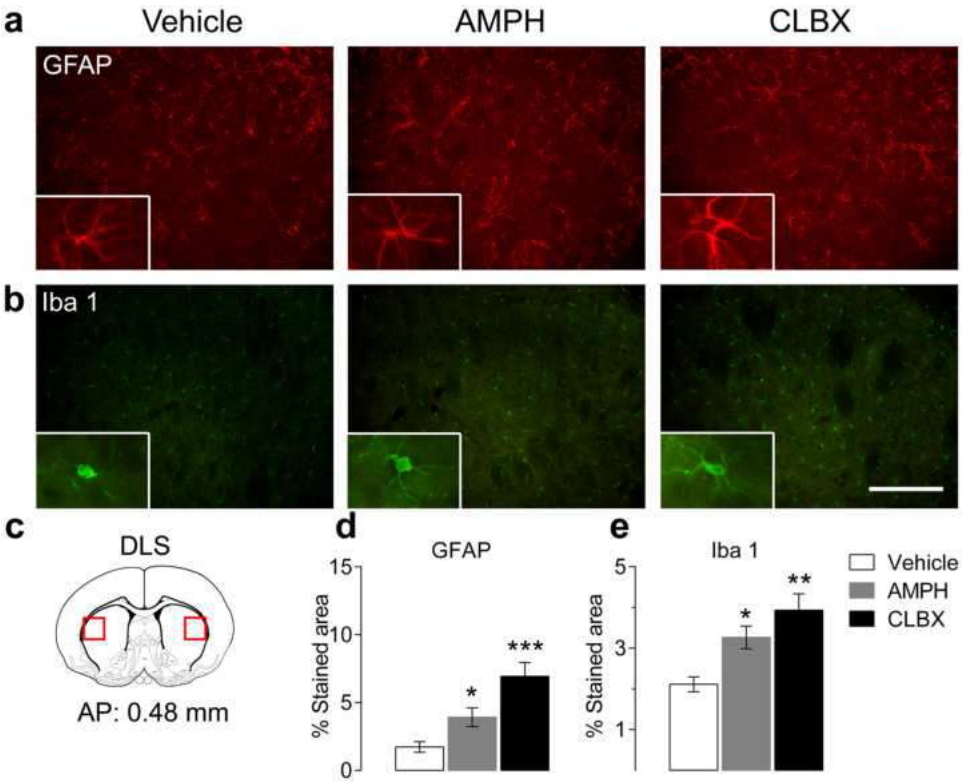

Fig. 7 Chronic clobenzorex or amphetamine administration induces GFAP and Iba-1 expression in the dorsolateral striatum. Photomicrographs of the dorsolateral striatum (DLS) section, stained with GFAP (**a**) and Iba-1 (**b**). (**c**) DLS scheme in a rat brain coronal section. Histograms show the proportional area of GFA-ir (**d**) and Iba-1-ir (**e**) staining. The values show mean ± SEM, $n = 4$ per group. Statistical analysis was conducted with a Kruskal-Wallis analysis and Dunn's multiple comparisons test. *$p < 0.05$ vs vehicle; **$p < 0.01$ vs vehicle; ***$p < 0.001$ vs vehicle. Bar indicates 200 µm

desensitization of DA receptors. The chronic administration of METH in rats has been shown to induce a decrease in D1 and D2 receptor levels (Segal et al. 2005; Thanos et al. 2017). Furthermore, a decrease in D2 receptor density has been demonstrated in non-human primates after chronic amphetamine treatment (Ginovart et al. 1999). The decreased D2 receptor levels induced by amphetamine seem to depend on the internalization mediated by arrestin-3 (Skinbjerg et al. 2010), while, in addition, a desensitization of the D2 receptor has been observed after chronic METH administration (Lacán et al. 2013).

It has been reported that one neurotoxic response to the administration of amphetamines or METH is glial reactivity, which triggers a neuroinflammation response (O'Callaghan et al. 1995; Thomas et al. 2004; Friend and Keefe 2013). METH increases reactive microglia levels in the striatum, hippocampus, cortex, and SNpc. METH also increases GFAP immunoreactivity in the striatum (Ares-Santos et al. 2014) and indusium griseum (Carmena et al. 2015). The present study found that chronic treatment with clobenzorex or amphetamine induces a significant increase in immunoreactivity to GFAP and Iba-1 in the dorsomedial and dorsolateral striatum, indicating the activation of astrocytes and microglia, respectively (Sofroniew and Vinters 2010; Ito et al. 1998). Although it is not clear exactly how amphetamines induce neuroinflammation, previous research has involved the activation of glutamatergic receptors and DA toxicity, as it is related to the induction of oxidative stress through the production of ROS, quinomas, and semiquinones (Thomas and Kuhn 2005; Kuhn et al. 2006; Shah et al. 2012; Northrop and Yamamoto 2014). Increased extracellular levels of DA and glutamate have been found in response to certain amphetamines (Nash and Yamamoto 1992). Therefore, it is reasonable to postulate that the activation of astrocytes and microglia induced by the administration of clobenzorex may be a consequence of the sustained release of DA, and probably glutamate, which promotes the formation of reactive oxygen species and generates oxidative stress.

Astrocytic reactivity is likely to be triggered at an early point during clobenzorex treatment, even before motor disturbances become apparent. Data obtained by our research group show that 15 days after treatment with clobenzorex, a significant increase in the number of GFAP-positive cells occurs (Supplementary Figure 1). These findings suggest that the motor dysfunction induced by chronic clobenzorex or amphetamine treatment may be related to the neuroinflammatory response observed in the striatum. It is important to mention that astrocytes, unlike microglia, play a protective role by increasing reduced glutathione (GSH) levels, increasing growth factor and molecule levels for axonal regeneration (Ares-Santos et al. 2014). These findings are probably related to the increased GFAP immunoreactivity observed 15 days after clobenzorex treatment. In summary, the results of the present study show that chronic treatment with clobenzorex at anorectic or amphetamine doses results in decreased motor function in rats. Although, under these

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treatment conditions, the nigrostriatal dopaminergic pathway was not itself affected, it did promote glial reactivity. These findings could have significant implications for the use of clobenzorex in the treatment of obesity, its consumption by truck drivers, and its deleterious effect on motor skills. However, more studies are required to explore the consequences of non-therapeutic and prolonged doses related to the abuse of clobenzorex and/or amphetamine (see Supplementary Figure Materials and Methods).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12640-021-00395-1.

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ORIGINAL ARTICLE

Design, synthesis, molecular docking and in vitro evaluation of benzothiazole derivatives as 11β-hydroxysteroid dehydrogenase type 1 inhibitors

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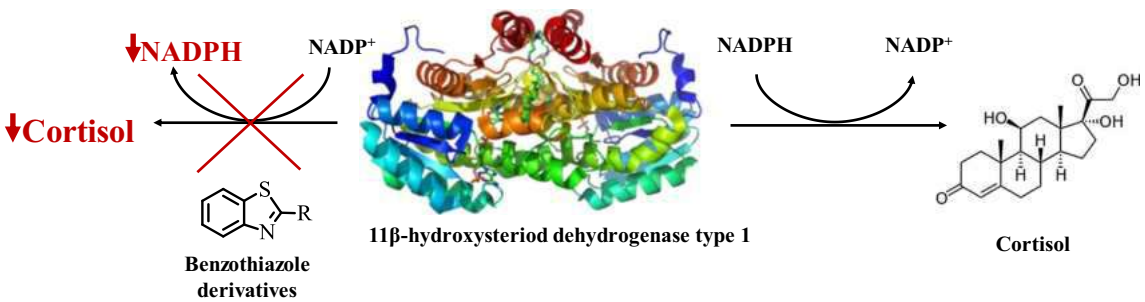
Abstract

11-Beta hydroxysteroid dehydrogenase type 1 (11β-HSD1) regulates cortisol levels mainly in adipose, hepatic and brain tissues. There is a relationship between the high activity of this enzyme and the development of obesity and metabolic disorders. The inhibition of 11β-HSD1 has been shown to attenuate the development of type 2 diabetes mellitus, insulin resistance, metabolic syndrome and other diseases mediated by excessive cortisol production. In this work, fifteen benzothiazole derivatives substituted with electron-withdrawing and electron-donating groups were designed to explore their affinity for 11β-HSD1 using in silico methods. The results show that (*E*)-5-((benzo[*d*]thiazol-2-ylimino)(methylthio)methylamino)-2-hydroxybenzoic acid (**C1**) has good physicochemical properties and favorable interactions with 11β-HSD1 through hydrogen bonding and hydrophobic interactions in the catalytic site formed by Y183, S170 and Y177. Furthermore, **C1** was synthesized and evaluated in vitro and ex vivo using clobenzorex (CLX) as a reference drug in obese Zucker rats. The in vitro results showed that **C1** was a better inhibitor of human 11β-HSD1 than CLX. The ex vivo assay results demonstrated that **C1** was capable of reducing 11β-HSD1 overexpression in mesenteric adipose tissue. Therefore, **C1** was able to decrease the activity and expression of 11β-HSD1 better than CLX.

Graphic abstract



Keywords 11β-Hydroxysteroid dehydrogenase type 1 · Cortisol · Benzothiazole derivatives · Enzyme inhibition · Obesity · Binding affinity

Introduction

The 11β-hydroxysteroid dehydrogenase isozymes (11β-HSDs) are microsomal enzymes that belong to the short-chain dehydrogenase/reductase family. They catalyze the intracellular conversion between physiologically active

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glucocorticoids (GCs) and their inert 11-keto counterparts in specific tissues [1]. The 11β-HSD isoform type 1 (11β-HSD1) is highly expressed in the liver, adipose tissue and the central nervous system. 11β-HSD1 acts as a NADPH-dependent reductase enzyme responsible for converting cortisone into the active glucocorticoid cortisol in humans by an acid–base mechanism [2, 3]. 11β-HSD2, present in the kidney and colon, is an exclusively NAD^+-dependent isoform that catalyzes the transformation of cortisol to inactive cortisone [4].

GC regulation by 11β-HSDs is of great importance because cortisol in humans is essential for the regulation of metabolic, immune and cardiovascular responses to stressful stimuli [5]. Recent investigations have shown that overexpression and elevated levels of 11β-HSD1 activity and the consequently high levels of cortisol are associated with a great number of metabolic disorders, such as obesity, insulin resistance, polycystic ovarian syndrome, osteoporosis and glaucoma, Cushing's syndrome and type 2 diabetes mellitus [6–8]. In addition, the correlation between 11β-HSD1 activity and obesity has been widely studied in humans and in genetically modified rodent models [9, 10]. Therefore, the reduction in intracellular GC levels via gene deletion or inhibition of 11β-HSD1 catalytic activity could be a treatment option against obesity [11]. In this way, clobenzorex (CLX) has been used for the treatment of obesity; it is capable of suppressing appetite and inhibiting 11β-HSD1. However, the use of CLX is prohibited in some countries because of addictive side effects and the increased probability of drug abuse [12, 13].

In previous studies, molecular docking showed that compounds, such as carbenoxolone (PDB 2BEL), resveratrol, SAR 184841 (PDB 4HX5) and PF877423 (PDB 3LZ6), have affinity by 11β-HSD1 due to moieties polyphenolics, adamantine sulfone, sulfonamide and thiazole [14–16].

Others analogs molecules have been developed as inhibitors, and their crystal structures coupled with 11β-HSD1 (PDB 3D4 N, 3D3E, 3BZU, 3BYZ, 2IRW, 2ILT, 2RBE, 3EY4, 4HFR and 4C7K) were found in Protein Data Bank (PDB) (Fig. 1). The crystal structures showed that chemical interactions with 11β-HSD1 are principally through hydrogen bonding between a heteroatom that acts as a hydrogen acceptor with an electronegative atom that has an hydrogen atom, such as oxygen or nitrogen, from the ligand. In addition, π–π interactions exist between an aromatic ring from the ligand with an aromatic ring from an amino acid residue, such as Y177, which suggested that these moieties may be important for interaction in the catalytic site on 11β-HSD1, similar to the substrate, corticosterone with PDB code 1Y5R (Fig. 1).

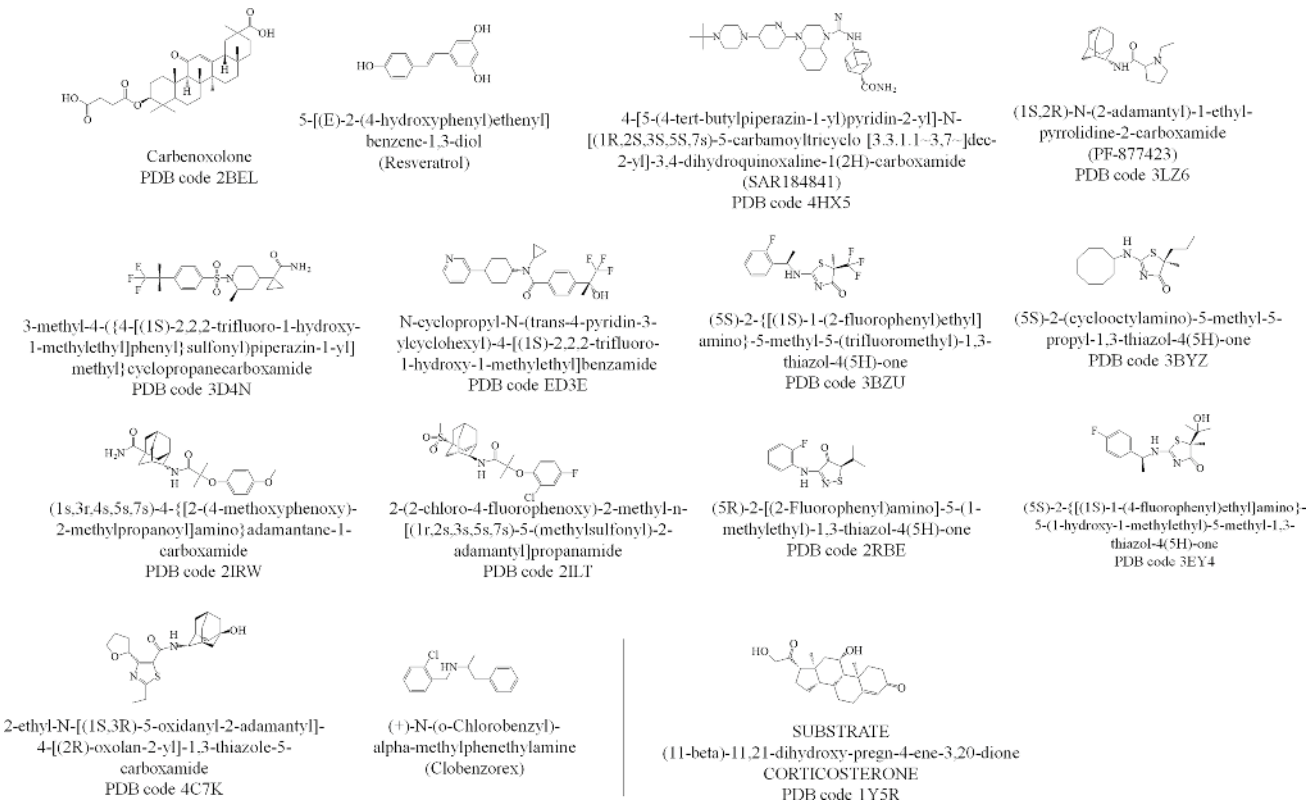

Fig. 1 Chemical structures of investigated 11β-HSD1 inhibitors

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Therefore, taking into account the chemical interactions that favor the recognition between 11β-HSD1 and its ligands, fifteen benzothiazole derivatives were designed bearing an aromatic ring, an amide group and additional hydrogen bond donor groups, with the aim of obtaining better 11β-HSD1 inhibitors. In addition, these compounds were monosubstituted with either electron-withdrawing or electron-donating groups on the aromatic benzothiazole group. The affinity of the target compounds for 11β-HSD1 was determined by molecular docking. In this approach, the best compound was selected considering its interaction energy, binding mode and in silico physicochemical and toxicological properties. Then, its 11β-HSD1 inhibitory activity was measured by an in vitro assay, and finally, the mRNA of 11β-HSD1 in mesenteric adipose tissue and serum cortisol were measured ex vivo in Zucker rats.

Materials and methods

In silico methods

Protein selection

The selection of protein sequences was performed after multiple alignment studies to select the consensus 11β-HSD1 sequence. The structure-based alignment program (STRAP) program available at http://www.bioinformatics.org/strap/ allowed us to determine that the sequence of 11β-HSD1 (PDB code: 2ILT) is highly conserved and mutations against the wild-type sequence (ID: AAK83653.1) are absent (http://www.ncbi.nlm.nih.gov/). Consequently, the 2ILT crystallized structure was selected for the evaluation of the protein–ligand complexes. This protein was obtained from RCSB Protein Data Bank coupled to adamantane sulfone-inhibitor-enzyme with a resolution of 2.30 Å.

Ligand selection

The development of 11β-HSD1 inhibitors could be a therapeutic strategy for the treatment of several metabolic pathologies. A docking study of (*E*)-5-((benzo[*d*]thiazol-2-ylimino)(methylthio)methylamino)-2-hydroxybenzoic acid (**C1**), a derivative of benzothiazole substituted with 5-aminosalicylic acid (5-ASA), into 11β-HSD1 was performed. **C1** was taken as the parent compound because it has the pharmacophore moieties to inhibit 11β-HSD1. Furthermore, two sets of **C1** analogs were designed and included for docking studies to 11β-HSD1 (Table 1). One set of compounds, **C3**–**C11**, contained electron-donating groups and a second set, **C12**–**C17**, contained electron-withdrawing groups at the 5, 6 or 7 position of the benzothiazole pharmacophore (Table 1 from supplementary material with their IUPAC names), with the purpose of testing these effects scoring the binding mode with 11β-HSD1. In addition, benzothiazole derivative substituted with L-tyrosine (**C2**) was tested to evaluate the effect of the 5-ASA moiety on 11β-HSD1 recognition [17].

Molecular docking of benzothiazole derivatives to 11β-HSD1

The human 11β-HSD1 enzyme (PDB code: 2ILT) was prepared by removing the co-crystallized ligand and water molecules. The polar hydrogen atoms were added with AutoDock Tools, and then partial Kollman charges were assigned. In contrast, CLX and the benzothiazole derivatives were drawn using ChemBioDraw Ultra 12.0 and prepared by the Avogadro program at pH = 7.4. The docking study was performed using the Lamarkian Genetic Algorithm as implemented in Auto Dock Tools 4.2.0. The center of the docking grid was located in a box 126 × 126 × 126 Å points with a grid spacing of 0.375 A^3 and an initial population of 100 individuals with 1×10^7 evaluations. The docking studies were validated using the 2ILT structure and the pattern ligand (NN1) in the presence of the cofactor $NADP^+$ by blind docking. Then, the most stabilizing interaction energy of each ligand with 2ILT was selected, and their interactions were visualized and analyzed using VMD v1.8.7.

Physicochemical and toxicological parameters

After docking studies, the physicochemical and toxicological parameters of CLX and the benzothiazole derivatives were estimated by Molinspiration (http://www.molinspiration.com/) and Osiris (http://www.organic-chemistry.org/prog/peo/) software, respectively. The physicochemical properties were based on Lipinski's rules (molecular weight (MW), number of hydrogen bond acceptors (HBA), number of hydrogen bond donors (HBD) and logarithmic octanol–water partition coefficient (Log *P*). The rule of five states that poor absorption or lipid membrane permeation is expected when MW > 500, HBD > 5, HBA > 10 or Log *P* > 5) [18]. The toxicological parameters included mutagenicity, tumorigenicity, irritancy and reproductive effects. Finally, the ΔG values and the binding pose in the catalytic site that was obtained from the blind docking were taken into account. Thus, compounds were ranked using the score values listed in Table 2, according to previous studies [19].

In vitro methods

11β-HSD1 inhibitory activity

The 11β-HSD1 inhibitory activity was measured by fluorescence as has been previously reported with certain

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Table 1 Ligand design of benzothiazole derivatives analogous to **C1**

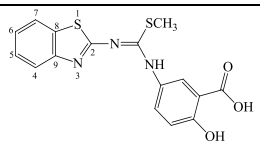
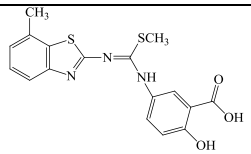
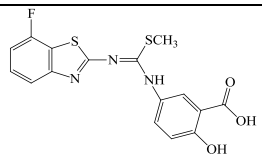
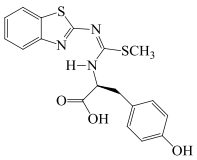
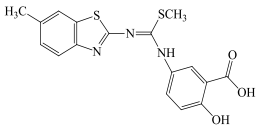
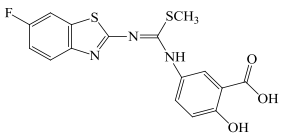
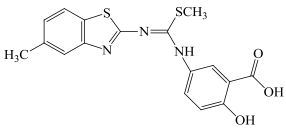
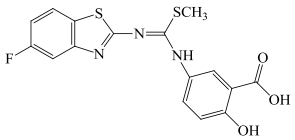
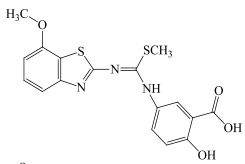
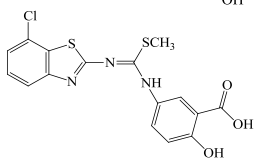
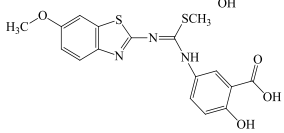
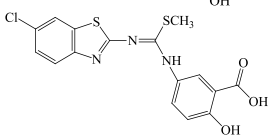
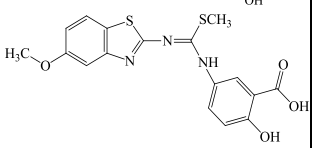
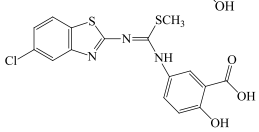
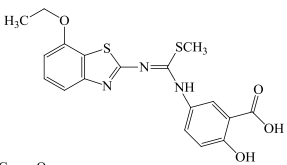
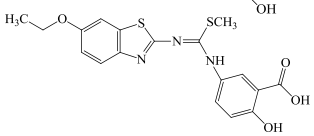
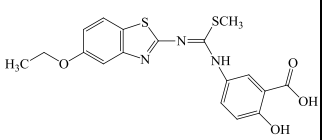
Parent benzothiazole		*Set 1: Benzothiazole derivative with electron-donating substituent*		*Set 2: Benzothiazole derivative with electron-withdrawing substituent*	
Ligand	*Structure*	*Ligand*	*Structure*	*Ligand*	*Structure*
C1		**C3**		**C12**	
C2		**C4**		**C13**	
		C5		**C14**	
		C6		**C15**	
		C7		**C16**	
		C8		**C17**	
		C9			
		C10			
		C11			

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Table 2 Score values assigned according to ΔG, binding pose on 11β-HSD1, physiochemical descriptors and toxicity risk assessment by in silico studies

Parameter			Assigned score		
ΔG	Absolute value				
Binding pose	Yes = 1		No = 0		
Log *P*	5–4 = 0	3.9–3 = 1	2.9–2 = 2	1.9–1 = 3	0.9–0 = 4
Polar surface	140–76 = 0		75–1 = 1		
MW	< 219 = 1	230–300 = 2	301–400 = 3	> 400 = 4	
HBA	< 10 = 1		> 10 = 0		
HBD	< 5 = 1		> 5 = 0		
Violations	1–4 = 1		5–7 = 2		
Rotatable bonds	Yes = 1		No = 1		
Mutagenesis	Yes = 1		No = 1		
Carcinogenesis	Yes = 1		No = 1		
Irritability	Yes = 1		No = 1		

The values of ΔG are expressed in kcal/mol^{-1} and the MW in Da

ΔG = Gibbs free energy, Log *P* = partition coefficient logarithm, MW = molecular weight expressed in Daltons, HBA = hydrogen bond acceptors, HBD = hydrogen bond donors

modifications [20]. Briefly, 10 µL of different concentrations of cortisol (0–200 mM) (Cayman Sigma, H0888) and 20 µL of dimethyl sulfoxide (DMSO) (0.00002%) in assay buffer (50 mM Tris pH 7.5, 150 mM NaCl 0.5 mM EDTA, 0.01% Brij35 and 200 mM $NADP^+$) were added in triplicate to a 96-well plate. The reactions were initiated by the addition of 170 µL of 11β-HSD1 (50 nM) (Cayman, 10007815) dissolved in assay buffer, followed by incubation for 1 h at 37 °C. The 11β-HSD1 activity was measured following NADPH production in an Infinite® M200 fluorometer from Tecan Trading AG (ex. 340 nm; em. 460 nm). Finally, the benzothiazole derivatives and CLX were dissolved in DMSO (0.00002%) and evaluated in assay buffer at a final concentration of 1 nM and a cortisol concentration of 200 µM. The percent inhibition was calculated by the following mathematical expression: % inhibition = $((Fluo_{ctl} - Fluo_{mta})/Fluo_{ctl}) \times 100$, where $Fluo_{ctl}$ = control fluorescence and $Fluo_{mta}$ = sample fluorescence.

Ex vivo methods

Animals, ethics statement and experimental protocols

Seven lean male HsdHlr:Zucker-Lepr rats and twenty-one obese male HsdHlr:Zucker-Lepr$^{fa/fa}$ rats were purchased from Harlan Laboratories, USA, at 9 weeks of age and housed in polysulfonate cages at 22 °C under a 12 h light/12 h dark cycle with lights on at 7:00 a.m. Food (Teklad Global Rodent Diet 2018S, Harlan®) and water were provided ad libitum.

The experimental design was based on four groups of seven rats each. Seven lean (Group I) and seven obese rats (Group II) received only vehicle (saline:ethanol; 50:50). Group III received CLX (10 mg/kg/day, 0.00003861 mol) in vehicle, and Group IV received compound **C1** (12 mg/kg/day, 0.00003343 mol). All the treatments were administered intragastrically for 28 days, after anesthetic administration of sodium pentobarbital (60 mg/kg, i.p.) the serum and mesenteric adipose tissue from the rats were obtained, and the cortisol and 11β-HSD1 expression levels were measured.

The animal experiments were conducted in accordance with the Mexican Official Standard NOM-062-ZOO-1999, Technical Specifications for Production, and Care and Use of Laboratory Animals. The animal protocol was approved by the Research Committee for the Care and Use of Laboratory Animals (CICUAL) of the Escuela Superior de Medicina-IPN (approval number: ESM.CICUAL-01/27-07-2015).

11β-HSD1 expression analysis

Total cellular RNA was extracted from frozen white adipose (WAT) tissue using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies). The total RNA sample (1 µg) was used as a template to make the cDNA using a reverse transcription kit (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics). The 11β-HSD1 mRNA levels in mesenteric adipose tissue were analyzed using a LightCycler TaqMan Master kit in a LightCycler® Nano Instrument (Roche Diagnostics) for real-time quantitative polymerase chain reaction (RT-qPCR). The reaction mixture was prepared according to the manufacturer's instructions (Roche Diagnostics, GmbH Mannheim, Germany). The thermal cycling parameters were as follows: 45 cycles of 10 s at 94 °C, 20 s at 60 °C and 5 s at 72 °C. The primer sequences used to amplify 11β-HSD1 were as follows: forward primer 5′-AAACAGAGCAATGGCAGCAT-3′ and reverse primer 5′-CAGAGGTTGGGTCATTTTCC-3′. The qPCR assay included a standard curve of four

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serial dilution points for the gene, and mRNA levels were calculated by using the comparative parameter threshold cycle (ct) method and normalized to the endogenous control 18S. The results were calculated as a percent of the mean level found in the control using the ΔCT method.

Serum cortisol levels

Serum cortisol levels were measured with ELISA using a cortisol EIA kit (Cayman Chemical Company, Cat.: 500360). Briefly, 100 µL of nonspecific binding (NSB) of the tracer was added per well in a 96-well plate. Next, the serum samples, an eight-point standard curve and maximum binding (Bo) were added each in a volume of 50 µL. Then, a cortisol acetylcholinesterase (AChE) tracer and a cortisol EIA monoclonal antibody were included. The plate was incubated overnight at 4 °C, and then reconstituted Ellman's reagent was added. The reaction was developed for 90 min, and the plate was read at 405 nm. The serum cortisol levels were obtained by interpolation from the standard curve using the plotted % B/Bo against the log of the cortisol concentration according to the recommendations of this kit.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SE) using GraphPad Prism 5.0 software. Statistical analyses were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered a significant difference between groups.

Results

In silico methods

Molecular docking of benzothiazole derivatives to 11β-HSD1

Figure 2a shows that the superimposed ligand NN1 redocked into the site active (Y183, S170 and K187 and polar residues T124, T222 and Y177) of the 11β-HSD1 enzyme is similar to the X-ray crystal structure-based binding pose of the control (PDB code: 2ILT). Furthermore, the interactions observed are similar to those observed in the crystal structure, with the ligand forming a hydrogen bond between its amide group and Y183 and S170 of the enzyme and a π–π interaction between the aromatic ring of the ligand with Y183 of the enzyme (Fig. 2b). Thus, these results suggest that the docking protocol is suitable for the in silico study of CLX and the benzothiazole derivatives.

Figure 2c shows the binding mode of CLX into 11β-HSD1. The chlorine moiety of CLX forms a hydrogen bond with the hydroxyl group of Y183 at a distance of 4.5 Å and a π–π interaction with the aromatic ring of Y177 at a distance of 3.9 Å. All other interactions between CLX and the active site residues of 11β-HSD1 consist of hydrophobic interactions between the benzyl and methyl groups of CLX with T124, T222 and S170 of the enzyme.

Compound **C1** ($\Delta G = -8.46$ kcal/mol) showed a better affinity for 11β-HSD1 than CLX ($\Delta G = -6.83$ kcal/mol) according to the docking studies. The amino group of **C1** makes contact with Y183 at a distance of 2.8 Å, and the sulfur atom of the thiazole ring makes contact with the hydrogen from S170 at a distance of 2.4 Å (Fig. 3a). Therefore, the thiazo group of **C1** was oriented toward the catalytic site of 11β-HSD1. Furthermore, the compound made π–π

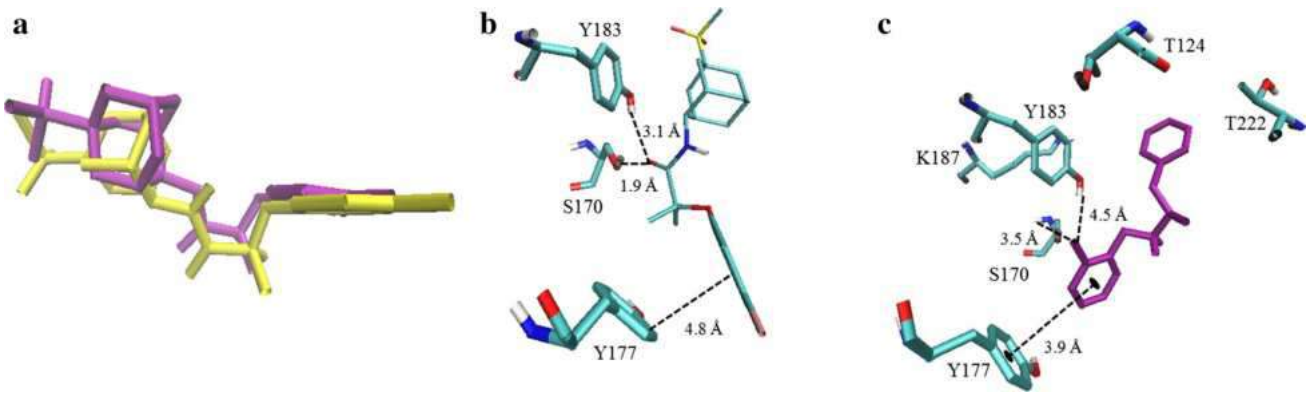

Fig. 2 Superimposed view of docked pose (purple) of the NN1 inhibitor with the crystal structure conformation (yellow) (**a**) and interaction analysis of lowest-energy docked pose of NN1 inhibitor (**b**). CLX with 11β-HSD1 (PDB code: 2LIT) (**c**) by blind docking procedure. (Color figure online)

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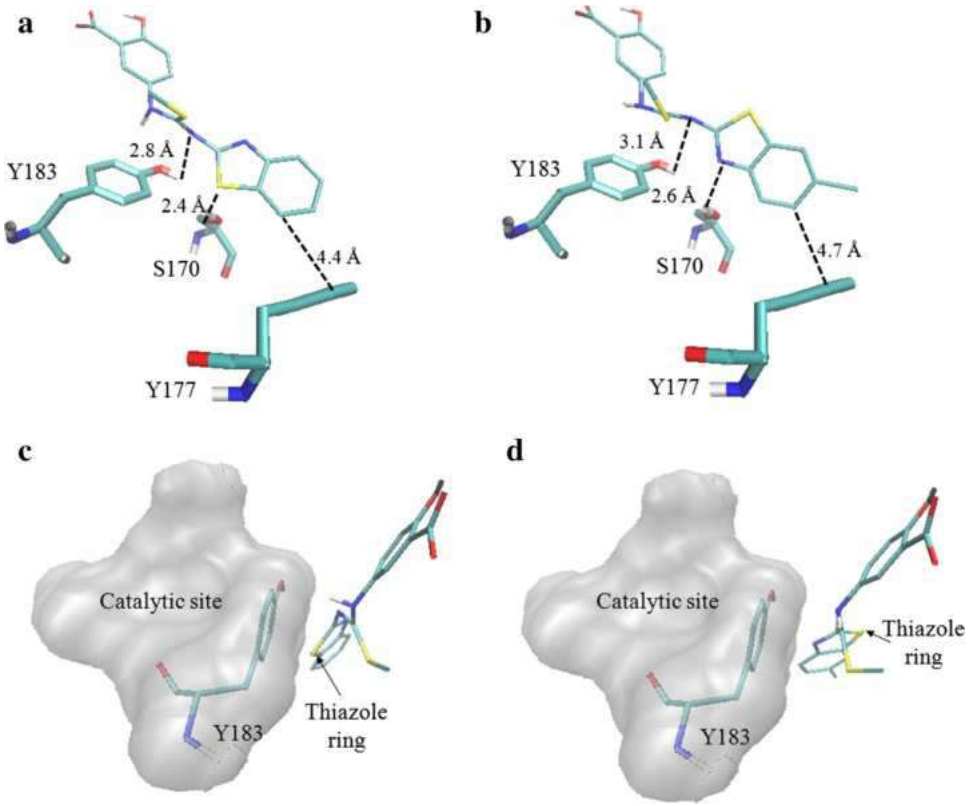

Fig. 3 Binding mode of benzothiazole derivatives with 11β-HSD1. Interaction analysis of lowest-energy docked pose of compounds **C1** (**a**) and **C16** (**b**) and orientation of thiazo moiety of compounds **C1** (**c**) and **C16** (**d**) with 2ILT under blind docking procedure

interactions between the aromatic ring of the benzothiazole group with Y177 at a distance of 4.4 Å (Fig. 3a).

Benzothiazole derivative **C16** showed a slightly better affinity for the enzyme ($\Delta G = -8.91$ kcal/mol), but its binding pose was different than **C1** on 2ILT. As shown in Fig. 2b, the thiazo group is oriented on the opposite side of the 11β-HSD1 catalytic site in comparison with **C1** (Fig. 3c); however, the compounds are able to establish similar interactions with Y183, Y177 and S170 and have similar distances of interaction, as depicted in Fig. 3d.

Moreover, the addition of substituted electron-donating and electron-withdrawing groups at positions 5 and 7 in compounds **C5**, **C6**, **C8**, **C12** and **C14** showed similar binding modes to **C1** (Fig. 4a) but different ΔG values (Table 2).

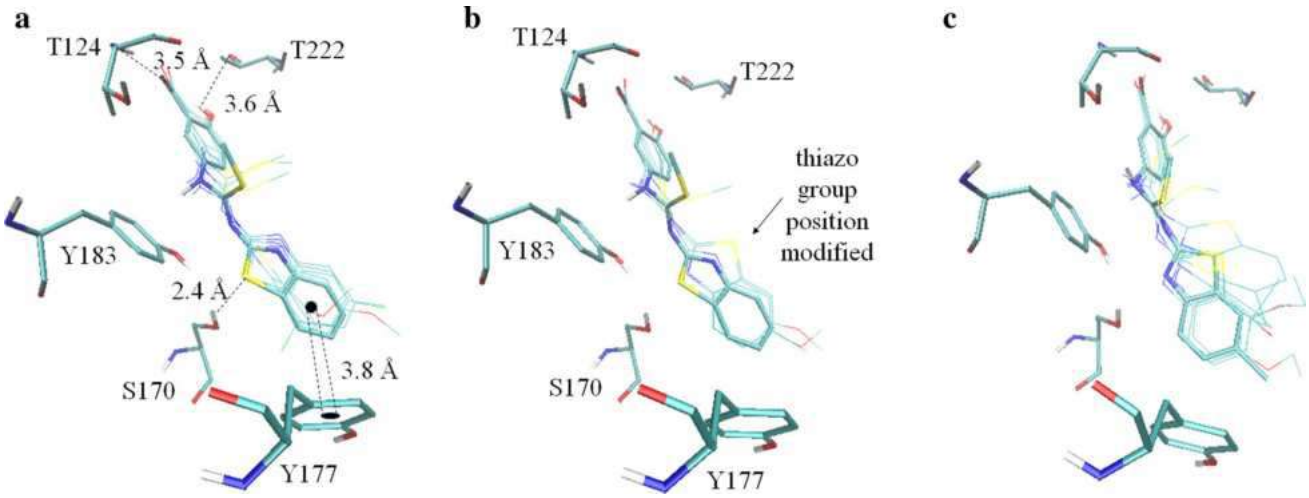

Fig. 4 Interaction analysis of lowest-energy docked pose of compounds **C1** (bonds) and **C5**, **C6**, **C8**, **C12** and **C14** (lines) (**a**) and representation of protein ligand interactions of compounds **C4**, **C7** and **C13** (lines) compared with **C1** (bonds) (**b**) and docked pose of compounds **C9**, **C10**, **C11**, **C15**, **C17** respect to **C16** with 2ILT by blind docking (**c**)

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The hydrogen bonds between the amine group of the **C1** analogs (**C5**, **C6**, **C8**, **C12** and **C14**) and the hydroxyl group of Y183 in 2ILT have a distance of 2.4 Å. The interaction between the sulfur of the thiazole ring with the hydrogen from the hydroxyl group of S170 is observed at 2.4 Å, and the π–π interaction is parallel-displaced with Y177 at 3.8 Å. In the hydrophobic region, the medium distances of 3.5 Å between the carbonyl group and hydroxyl of the 5-ASA moiety of the different benzothiazole derivatives are observed with T124 and T222 (Fig. 4a). However, the addition of an electron-donating group (CH_3) in the 5-position of **C5** produced better recognition to the 11β-HSD1 enzyme compared to **C14**, in which the substituent is replaced by an electron-withdrawing group. In contrast, the addition of a CH_3 or F in the 6-position in compounds **C4**, **C7** and **C13** negatively affected the binding mode and the ΔG values (Fig. 4b and Table 3).

In contrast, substitution with groups with higher atomic radii (OCH_2CH_3 and Cl) at positions 5, 6 and 7 in compounds **C9**, **C10**, **C11**, **C15** and **C17** confers a recognition similar to **C16** (Fig. 4c). These results suggest that substitution with electron-donating groups could be better than with electron-withdrawing groups and the position of the substituent affects the binding mode and ΔG values of the compound with the 11β-HSD1 enzyme.

The compound **C2**, substituted with L-tyr as the phenolic moiety, shows a lower affinity than **C1** and different binding poses. The thiazo group of **C2** is oriented toward the hydrophobic site on 2ILT. The benzene ring of L-tyr interacted through π–π bonds with Y177. Additionally, the hydrogens of the amino group and the phenolic hydroxyl interacted with S170 and L217 at distances of 4.2 and 3.6 Å, respectively (Fig. 5). The benzothiazole ring is oriented in the direction of T124 and T222 instead of the carboxyl group from 5-ASA as in **C1**, showing that the carboxyl group in the 5-ASA structure is important to maintain interactions with T124 and T222 (Fig. 4).

Physicochemical and toxicological parameters to select the best compound

The five Lipinski rules are widely accepted as physicochemical parameters for predicting drug-likeness descriptors for drug design. These parameters are in Table 3 for benzothiazole derivatives **C1**–**C17** and CLX. According to Lipinski's rules, a compound with high drug-likeliness satisfies the following criteria: molecular weight (MW) lower than 500 Daltons (Da), a partition octanol–water coefficient (Log) less than 5, the compound should not have more than 5 hydrogen bond donors or 10 hydrogen bond acceptors. The benzothiazole derivatives and CLX meet Lipinski's rules and drug score. However, compounds **C15–C17** show violations due to Log values above 5. These results suggest poor oral bioavailability. However, the benzothiazole derivatives do not show risk to human health considering the four main parameters of analysis: mutagenicity, tumorigenicity, irritability and reproductive effectiveness, according to the Osiris predictor.

Compounds **C1** and **C2** were chosen for in vitro evaluation. The first showed one of the best physicochemical and toxicological profiles (final score value of 18.5, Table 3) compared to the properties of the analogs (**C3**–**C17**). Additionally, compound **C1** had been evaluated previously as an antioxidant, and its synthesis and characterization have been reported [17]. In contrast, the second compound had a better final score value (20.1) than **C1** but a different binding pose. The synthesis of compounds **C1** and **C2** was performed by nucleophilic substitution following a reported procedure [17].

In vitro methods

11β-HSD1 inhibitory activity

The 11β-HSD1 dehydrogenase activity in the presence of different concentrations of cortisol and at a fixed level of $NADP^+$ is shown in Fig. 6a. The hyperbolic response of human 11β-HSD1 activity corresponds to Michaelis–Menten-type kinetics. The maximum catalytic rate (Vmax = 3.6 μM/min) and apparent affinity, through measurement of the Michaelis–Menten constant (Km = 193 μM), were determined according to the model of Lineweaver–Burk (Fig. 6b).

During the 11β-HSD1 dehydrogenase activity assay, the NADPH concentration increases due to the reduction of $NADP^+$. In the presence of CLX, a decrease in NADPH production was observed (Fig. 6c), which suggests 11β-HSD1 inhibition. Moreover, the conversion of $NADP^+$ at stoichiometric concentrations (0.001 μM) was minimal in the presence of **C1** compared with CLX and compound **C2** (Fig. 6c).

In addition, Fig. 6d shows that compound **C1** has a higher percent 11β-HSD1 inhibition (% inhibition) compared to CLX and **C2**, suggesting that **C1** is a better inhibitory agent. These results correlated with the in silico studies in which **C1** was a better 11β-HSD1 inhibitor than CLX and **C2**. Then, **C1** and CLX were analyzed in an obesity model using Zucker rats. It is important to mention that CLX was used in this study because it is employed during obesity treatment despite its side effects, it has been reported as an 11β-HSD1 inhibitor, and it shares a similar chemical structure with the benzothiazole derivatives, such as two aromatic rings and an electronegative atom acting as a hydrogen bond donor.

Table 3 Final score of benzothiazole derivatives based on each physicochemical and toxicity risk parameters obtained by in silico methods

Compound	$-\Delta G$	Binding pose	Log *P*	Score	Polar surface	Score	MW	Score	HBD	Score	HBA	Score	Violations	Rotable bonds	Score	*M*	*C*	*I*	*R*	Final score
C1	−8.46	1	4.49	0	94.81	0	359.43	1	6	1	3	1	0	5	2	1	1	1	1	18.5
C2	−8.05	0	1.89	3	94.81	0	387.49	1	6	1	3	1	0	7	2	1	1	1	1	20.1
C3	−8.48	0	4.79	0	94.81	0	373.46	1	6	1	3	1	0	5	2	1	1	1	1	17.5
C4	−8.45	0	4.82	0	94.81	0	373.47	1	6	1	3	1	0	5	2	1	1	1	1	17.5
C5	−8.68	1	4.82	0	94.81	0	373.48	1	6	1	3	1	0	5	2	1	1	1	1	18.7
C6	−8.21	1	4.40	0	104.04	0	389.46	1	7	1	3	1	0	6	2	1	1	1	1	18.2
C7	−8.23	0	4.43	0	104.04	0	389.46	1	7	1	3	1	0	6	2	1	1	1	1	17.2
C8	−8.09	1	4.43	0	104.04	0	389.46	1	7	1	3	1	0	6	2	1	1	1	1	18.1
C9	−8.21	0	4.78	0	104.04	0	403.49	0	7	1	3	1	0	7	2	1	1	1	1	16.2
C10	−8.46	0	4.80	0	104.04	0	403.50	0	7	1	3	1	0	7	2	1	1	1	1	16.5
C11	−7.73	0	4.80	0	104.04	0	403.51	0	7	1	3	1	0	7	2	1	1	1	1	15.7
C12	−8.10	1	4.51	0	94.81	0	377.42	1	6	1	3	1	0	5	2	1	1	1	1	18.1
C13	−8.19	0	4.53	0	94.81	0	377.43	1	6	1	3	1	0	5	2	1	1	1	1	17.2
C14	−8.39	1	4.53	0	94.81	0	377.44	1	6	1	3	1	0	5	2	1	1	1	1	18.4
C15	−8.34	0	5.02	0	94.81	0	393.88	1	6	1	3	1	1	5	2	1	1	1	1	16.3
C16	−8.91	0	5.05	0	94.81	0	393.88	1	6	1	3	1	1	5	2	1	1	1	1	16.9
C17	−8.28	0	5.05	0	94.81	0	393.88	1	6	1	3	1	1	5	2	1	1	1	1	16.3
CLX	−6.83	0	4.26	0	12.00	1	259.78	1	1	1	1	1	0	5	2	1	1	1	1	16.8

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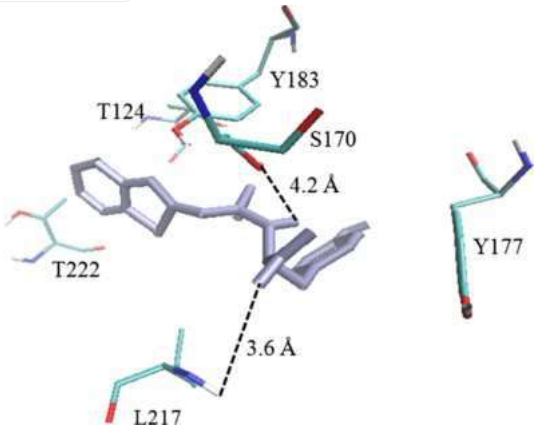

Fig. 5 Interaction analysis of lowest-energy docked pose of compounds **C2** on 2ILT by blind docking procedure

Ex vivo methods

11β-HSD1 expression analysis

The 11β-HSD1 expression in response to an obese state was examined. In Fig. 7, it is possible to observe that the adipose tissue samples from the obese rats (Group II) treated with vehicle (saline:ethanol solution) had high levels of 11β-HSD1 expressed compared with the lean rats (Group I). However, in groups III and IV, treated with CLX and compound **C1**, respectively, the mRNA expression of 11β-HSD1 after 28 days was significantly diminished.

Serum cortisol levels

The serum cortisol levels in control Group I (513 ± 68 pg/mL) were not different from the levels found in the obese rats Group II subjected to the same treatment (543 ± 93 pg/mL) (Fig. 8). In contrast, treatment with CLX and **C1** can decrease cortisol levels, although not significantly, compared

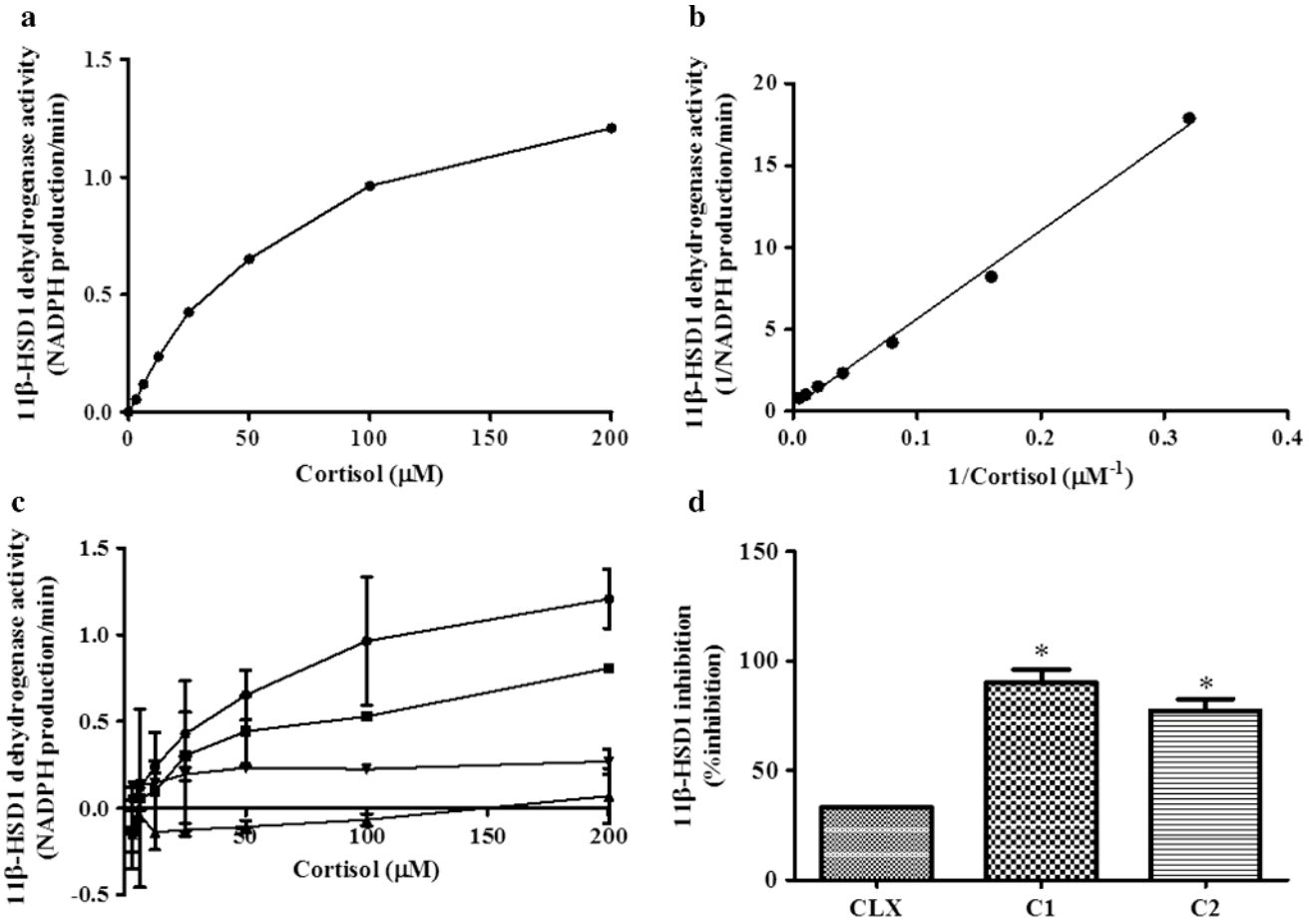

Fig. 6 Michaelis–Menten kinetics of 11β-HSD1 dehydrogenase activity ($n = 3$) (**a**); double reciprocal plot of the kinetics data (**b**); effect of CLX (filled square), benzothiazole derivatives **C1** (filled triangle) and **C2** (filled inverted triangle) on 11β-HSD1 dehydrogenase activity (filled circle) (**c**); and % inhibition of 11β-HSD1 by **C1** and **C2** ($n = 6$) against CLX at 200 μM cortisol (**d**). *$P < 0.05$

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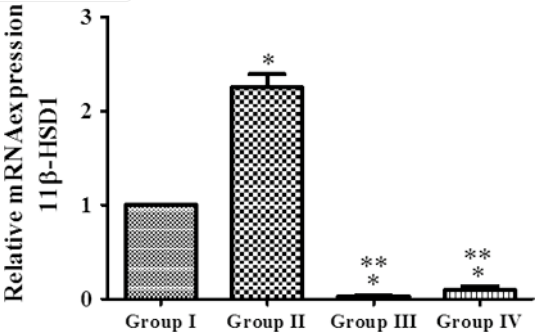

Fig. 7 Relative 11β-HSD1 mRNA expression by RT-PCR. Lean rats (Group I), obese rats treated with vehicle saline:ethanol solution (Group II), obese rats treated with CLX (Group III), obese rats treated with compound C1 (Group IV). *$P<0.05$ against Group I, **$P<0.05$ against Group II

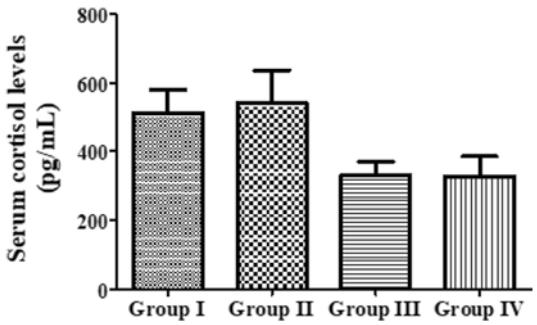

Fig. 8 Serum cortisol levels in four different experimental groups after 28 days of treatment ($n=5$). Lean rats (Group I), obese rats treated with vehicle saline:ethanol solution (Group II), obese rats treated with CLX (Group III), obese rats treated with compound C1 (Group IV)

to the group of obese rats treated only with vehicle (Group II) after 28 days of treatment.

Discussion

Cortisol is a glucocorticoid hormone that promotes the conversion of proteins and lipids into glucose. Cortisol formation from cortisone is regulated by 11β-HSD1 catalytic activity [21]. The catalytic site of 11β-HSD1 is formed by the amino acid residues S170, Y183 and K187. Thus, cortisol formation is mediated by interactions between the ketone group of cortisone and S170. The residue Y183 induces the protonation of the ketone moiety of the substrate, while K187 interacts with nicotinamide ring cofactor NADPH, allowing the hydride transfer from NADPH to the carbon atom of the ketone group to produce cortisol [22].

Carbenoxolone was the first commercial inhibitor of 11β-HSD1 assayed in vivo; however, its use was discontinued due to its side effects [23]. Several compounds have been developed and evaluated as potential 11β-HSD1 inhibitors [24–26]. These 11β-HSD1 inhibitors include functional groups capable of establishing hydrogen bonds with the catalytic site of the enzyme as well as terminal hydrophobic moieties and aromatic-substituted rings. However, these molecules have shown inconclusive results [27]. Therefore, in this study, two sets of benzothiazole derivatives substituted with 5-ASA and different electron-withdrawing or electron-donating substituents (**C1, C3–C17**) were studied, and a benzothiazole derivative substituted with L-tyrosine (**C2**) was included to explore the effects of 5-ASA.

The docking results show that benzothiazole derivatives substituted with electron-donating groups have interactions with Y183, T124, T222 and S170 in the 2LIT structure. Furthermore, the results demonstrate that the position of the substituent on the benzothiazole ring is also important, suggesting that hindrance effects affect the binding mode in 2ILT compared to **C1**. For example, the hydrogen of the amine group in the compounds containing F, CH_3 or OCH_3 groups located at 5 and 7 positions (**C3**, **C5**, **C6**, **C8**, **C12** and **C14**) generates a hydrogen bond with S170, T124 and T222 at the same time that the π–π interaction is generated with Y177, and some of these interactions have been reported for similar compounds [28, 29]. Moreover, compounds **C9**–**C11** and **C15–C17** substituted with larger groups (Cl or OCH_2CH_3) in the 5, 6 or 7 positions and compounds substituted in the 6-position with F, CH_3 or OCH_3, such as **C4**, **C7** and **C13**, have different recognitions in the X-ray structure of 2ILT, where the thiazo group is oriented opposite to the catalytic site, indicating that electronic effects and substituent position could influence molecular recognition. This suggests that to design better 11β-HSD1 inhibitors, the compound should be substituted by an electron-donating group at the five positions of the benzothiazole ring.

In addition, the hydrogen atom of the amine group of the benzothiazole derivatives with 5-ASA acts as a donor and forms a hydrogen bond with the oxygen atom on the side chain of Y183, which is stronger in **C1** and **C5** because the bond distance is minor compared with other benzothiazole derivatives; this suggests that the hydrogen atom of the amine group prevents the release of protons and the transfer of hydride, which consequently prevents the formation of cortisol, suggesting inhibition of the enzyme [22]. Additionally, the benzothiazole derivatives **C1** and **C5** show a parallel-displaced π–π interaction with Y177, suggesting the importance of this residue for molecular recognition, along with residues T124 and T222, which make hydrogen bonding interactions with the COOH and OH groups of the 5-ASA moiety from **C1** and **C5**. Therefore, it is possible that the amine, phenol and benzothiazole groups incorporated in the designed compounds are good pharmacophores to

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inhibit the catalytic site of 11β-HSD1 [30] because a balance between the potential donors/acceptors of hydrogen bonds and hydrophobic atoms is achieved.

The substitution of 5-ASA by L-tyr in compound **C2** affected the affinity to the 11β-HSD1 enzyme and also the physicochemical properties of the compound. However, **C2** showed a very low Log *P* (Log *P*= 1.89), which could affect its diffusion through the membrane; therefore, it was interesting to synthesize **C1** and **C2**, as previously reported [17], to study their effect on 11β-HSD1 inhibition.

The 11β-HSD1 kinetics showed Michaelis–Menten behavior according to previous studies. Compounds **C1** and **C2** showed a greater percent inhibition than CLX. The inhibitory effect of **C1** was greater compared with **C2**, which was correlated with its better affinity obtained by the in silico studies. It is important to mention that the affinities obtained for **C1** and **C2** were similar to those obtained for designed inhibitors with oleanan and ursan scaffolds [31].

High 11β-HSD1 activity provokes increased GC production in adipose tissue, which is related to several diseases. 11β-HSD1 gene expression is increased in adipose tissue in obese Zucker rats and in the tissue of obese patients, as demonstrated by measurements. The 11β-HSD1 mRNA level was measured using real-time PCR, showing that 11β-HSD1 expression increased in the samples of mesenteric adipose tissue of obese Zucker rats compared to lean animals, as has been reported in previous works [32, 33]. The administration of CLX and **C1** diminished the 11β-HSD1 mRNA levels. Therefore, both compounds not only modify 11β-HSD1 activity, as was demonstrated in vitro, but they can also modify the transcription of the 11β-HSD1 gene. This last effect has been reported with rosiglitazone, which diminishes 11β-HSD1 expression [34]. However, no significant changes were observed in serum cortisol levels, probably because of the dysregulation of cortisol observed from the obesity results due to alterations in 11β-HSD1 in both the liver and mesenteric adipose tissues; these levels are greater than the systemic level, which has an adrenal origin. These results suggest that although **C1** and CLX inhibit 11β-HSD1 expression, they did not attenuate the hypothalamic–pituitary–adrenal (HPA) effect [35–37]. However, in this work, the cortisol level in adipose tissue was not measured, which could be done in future works, and glucose metabolism could also be studied.

Conclusion

In conclusion, **C1** is a human 11β-HSD1 inhibitor that was able to diminish the expression of the 11β-HSD1 enzyme. In silico studies suggest that this compound is capable of interacting with the active site of the 11β-HSD1 enzyme, as demonstrated by in vitro studies. The substitution of the benzothiazole produced slight changes in the ΔG values and different binding modes, suggesting that steric and electronic effects influence recognition, and better 11β-HSD1 inhibitors could be obtained through substitution of the 5 position of the benzothiazole ring with electron-donating groups.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Exploration of interaction interface of TRKβ/BDNF through fingerprint analysis to disinter potential agonists

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Abstract

Tyrosine Kinase beta (TRKβ), is a type I membrane receptor which plays a major role in various signalling pathways. TRKβ was found to be upregulated in various cancers and contrastingly downregulated in various neurodegenerative disorders. Hitherto, contemporary drug research is oriented towards discovery of TRKβ inhibitors, thus neglecting the development of TRKβ agonists. This research is aimed at identifying FDA approved drugs exhibiting repurposable potential as TRKβ agonists by mapping them with fingerprints of the BDNF/TRKβ interaction interface. Initially, crucial interacting residues were retrieved and a receptor grid was generated around it. TRKβ agonists were retrieved from literature search and a drug library was created for each agonist based on its structural and side effect similarities. Subsequently, molecular docking and dynamics were performed for each library to identify the drugs possessing affinity towards the binding pocket of TRKβ. The study revealed molecular interactions of Perospirone, Droperidol, Urapidil, and Clobenzorex with the crucial amino acids lining the active binding pocket of TRKβ. Subsequent network pharmacological analysis of the above drugs revealed their interactions with key proteins involved in neurotransmitter signalling pathways. Clobenzorex displayed high stability in dynamics simulation and therefore this drug is recommended for further experimental evaluations to attain better mechanistic insights and predict its implications in correcting neuropathological aberrations. This study's focus on the interaction interface between TRKβ and BDNF, combined with the utilization of fingerprint analysis for drug repurposing, contributes to our understanding of neurotrophic signalling and holds potential for identifying new therapeutic options for neurological disorders.

Keywords TRKβ · Neurological disorders · Drug repurposing · Clobenzorex · Network pharmacology

Introduction

Tyrosine Kinase beta (TRKβ) (EC 2.7.10.1), also known as Neurotrophic Receptor Tyrosine Kinase 2 (NTRK2) (NCBI gene id: 4915) is a type 1 transmembrane protein (822 amino acid) coded by TRKβ gene located at chromosome 9q21.33 [1]. It is a member of the family of Receptor Tyrosine Kinases (RTKs), which are activated by interacting with their associated ligands. As a result, RTKs autophosphorylate tyrosine residues on the receptors as well as phosphorylate downstream signaling proteins to transmit extracellular signals to the cytoplasm. RTK activation initiates a number of intracellular signaling pathways that alter cellular functions such as cell growth, differentiation, migration, and metabolism. Other members of the RTK family include insulin receptors as well as other growth factor receptors such as epidermal growth factor, fibroblast growth factor,

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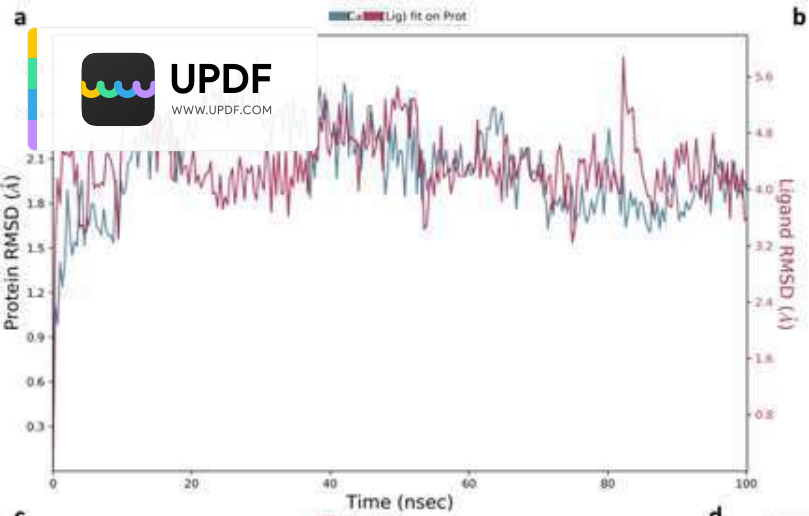

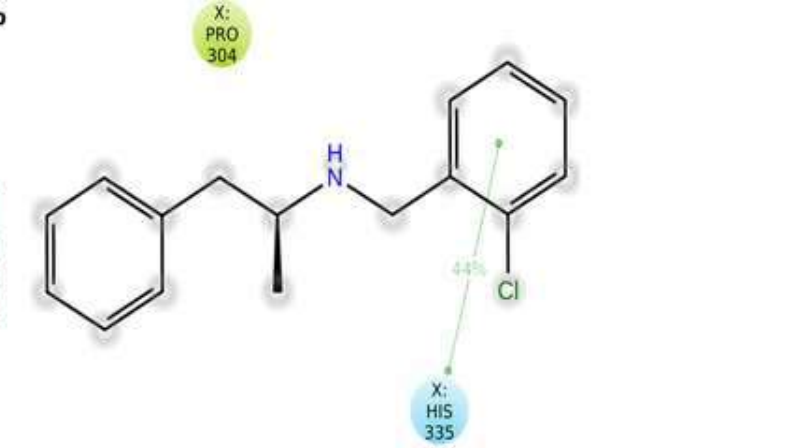

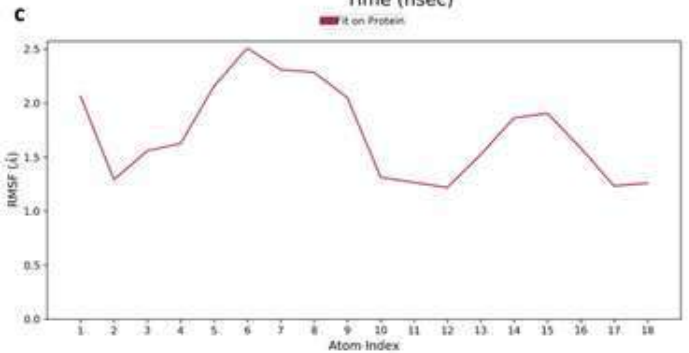

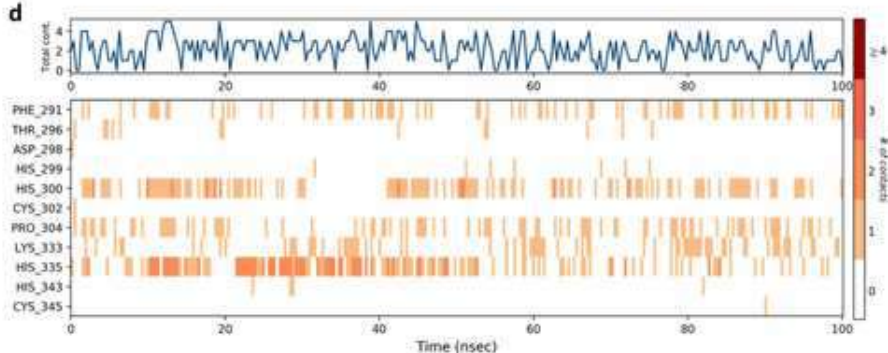

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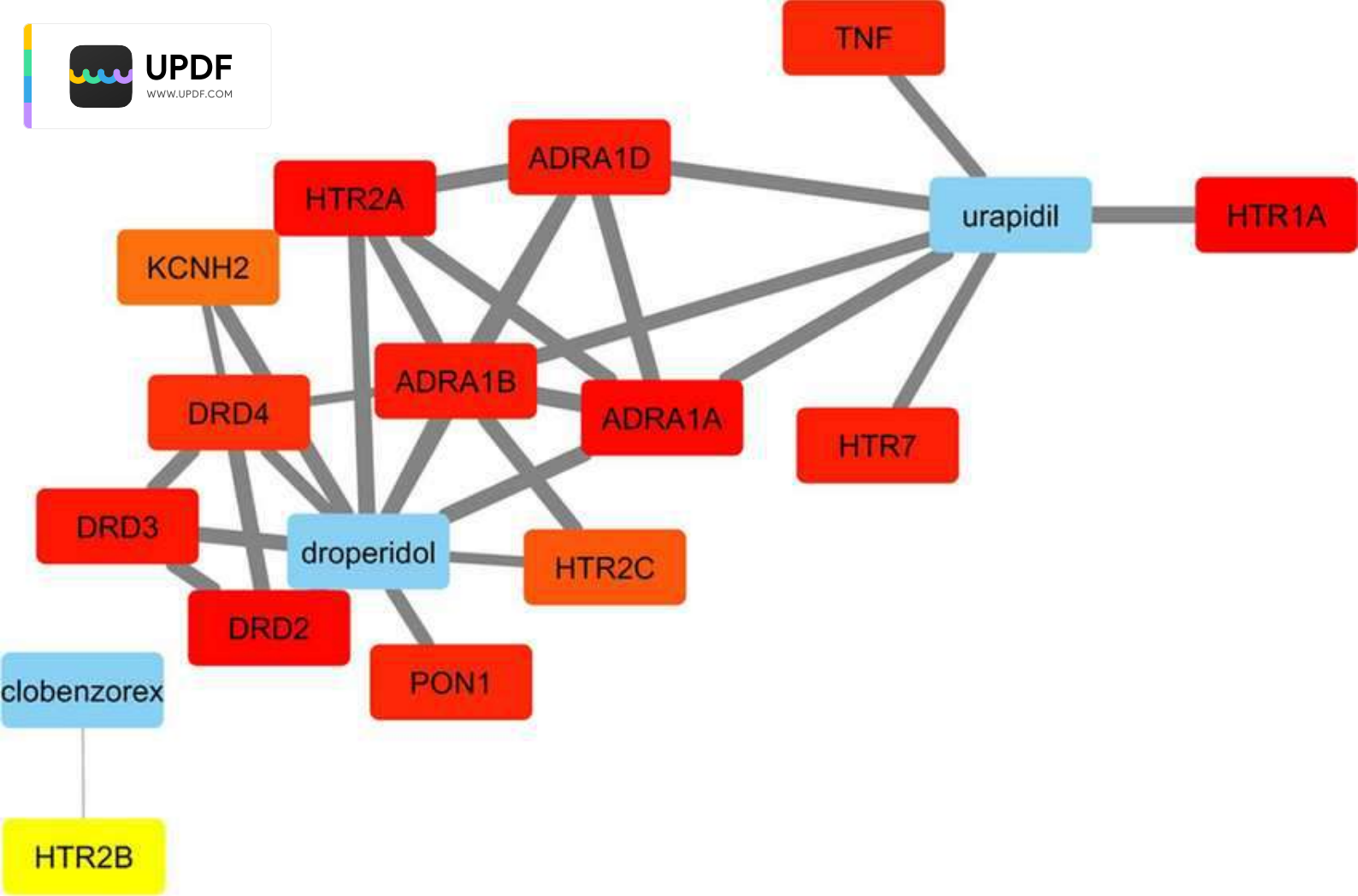

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Synthèse du Chlorhydrate de (+)-N-(o-Chlorobenzyl) α-Methyl Phénéthylamine marqué en position 7 par ^{14}C* (Chlorhydrate de Clobenzorex)**

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SUMMARY

The synthesis of (+)-N-(o-chlorobenzyl) α-methyl phenethylamine hydrochloride (Clobenzorex hydrochloride) labelled at position 7 with C^{14} is described. (±)-amphetamine was prepared in 5 steps from benzoic acid-7-^{14}C. Resolution of (±)-amphetamine with use of (+)-tartaric acid and with modification of the ratio of the two isomeres by adding a quantity of non radioactive (+)-amphetamine similar to that of labelled racemic amphetamine gave (+)-amphetamine (+)-tartrate with high optical purity and a very good yield after only one crystallization. The specific radioactivity of the (+)-amphetamine obtained was 18 mCi/mM. The same procedure provided the (−)-amphetamine-7-^{14}C from the mother liquors of the previous operation by adding (−)-tartaric acid. Its specific activity was 15 mCi/mM. The (+)-N-(o-chlorobenzyl) α-methyl phenethylamine hydrochloride-7-^{14}C was obtained by condensation of the (+)-amphetamine-7-^{14}C with o-chlorobenzaldehyde followed by a reduction with sodium borohydride. The final product had a specific activity of 17.2 mCi/mM and a radiochemical purity of about 99 %.

* Ce travail a été réalisé au Service des Molécules Marquées, CEN, SACLAY, dirigé par le Dr L. Pichat. Les auteurs lui expriment ici leurs remerciements pour ses judicieux conseils.

** Dénomination commune internationale recommandée.

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RÉSUMÉ

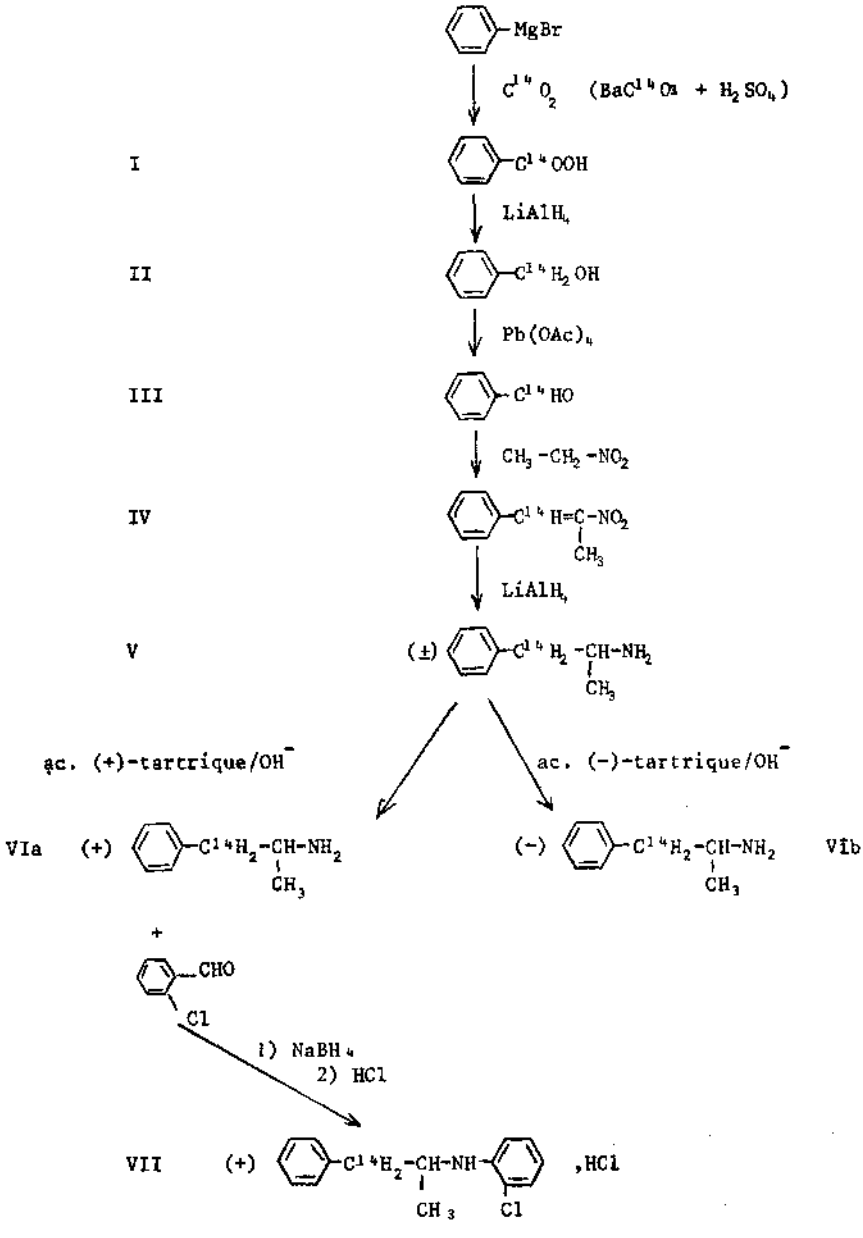
Nous décrivons la synthèse du chlorhydrate de (+)-N-(o-chlorobenzyl) α-méthyl phénéthylamine (Chlorhydrate de Clobenzorex) marqué en position 7 par le ^{14}C. Nous avons préparé la (±)-amphétamine ^{14}C en 5 étapes à partir de l'acide benzoïque ^{14}C-7. Son dédoublement optique a été effectué par l'intermédiaire du sel avec l'acide (+)-tartrique. En modifiant avant la salification la proportion des deux isomères par addition à la (±)-amphétamine ^{14}C-7 d'une égale quantité de (+)-amphétamine non radioactive, nous avons obtenu, après une seule cristallisation, le (+)-tartrate de (+)-amphétamine ^{14}C-7 d'une excellente pureté optique et avec un très bon rendement. Ce sel a conduit à la (+)-amphétamine ^{14}C-7 par condensation de la (+)-amphétamine ^{14}C-7 avec l'o-chloro-appliqué aux eaux-mères de cristallisation a permis, par addition d'acide (−)-tartrique, d'isoler la (−)-amphétamine ^{14}C-7 d'une activité spécifique de 15 mCi/mM. Nous avons synthétisé le chlorhydrate de (+)-N-(o-chlorobenzyl) α-méthyl phénéthylamine ^{14}C-7 par condensation de la (+)-amphétamine ^{14}C-7 avec l'o-chlorobenzaldéhyde, suivie d'une réduction par le borohydrure de sodium. Le produit final a une activité spécifique de 17,2 mCi/mM et une pureté radiochimique de l'ordre de 99 %.

Le métabolisme chez l'homme et chez l'animal de l'amphétamine et de certains de ses dérivés, pour la plupart anorexigènes, a fait l'objet de nombreuses études dont nous avons récemment publié l'ensemble des résultats (Benakis *et al.*, 1968).

Dans une série décrite en 1966 par Boissier *et al.*, plusieurs composés ont montré une activité anorexigène particulièrement intéressante associée à une action psychoanaleptique très réduite, en particulier le chlorhydrate de (+)-N-(o-chlorobenzyl) α-méthyl phénéthylamine (Chlorhydrate de Clobenzorex). Pour étudier le métabolisme de ce dernier produit, nous avons préparé cette molécule marquée par le ^{14}C en position 7 avec une activité spécifique élevée en vue des études de localisation par autohistoradiographie.

Nous décrivons cette synthèse qui comporte les étapes suivantes explicitées dans le tableau :

I — Acide benzoïque ^{14}C-7.
II — Alcool benzylique ^{14}C-7.
III — Benzaldéhyde ^{14}C-7.
IV — Phényl-1 nitro-2 propène ^{14}C-1.
V — (±)-amphétamine ^{14}C-7.
VI — Dédoublement optique : (+)-amphétamine ^{14}C-7 et (−)-amphétamine ^{14}C-7.
VII — Chlorhydrate de (+)-N-(o-chlorobenzyl)α-méthylphénéthylamine^{14}C-7.


MgBr
$C^{14}O_2$ ($BaC^{14}O_3$ + H_2SO_4)
I
$C^{14}OOH$
$LiAlH_4$
II
$C^{14}H_2OH$
$Pb(OAc)_4$
III
$C^{14}HO$
$CH_3-CH_2-NO_2$
IV
$C^{14}H=C(CH_3)-NO_2$
$LiAlH_4$
V
(±) $C^{14}H_2-CH(CH_3)-NH_2$
ac. (+)-tartrique/OH^-
ac. (-)-tartrique/OH^-
VIa (+) $C^{14}H_2-CH(CH_3)-NH_2$
(-) $C^{14}H_2-CH(CH_3)-NH_2$ VIb
+
CHO
Cl
1) $NaBH_4$
2) HCl
VII (+) $C^{14}H_2-CH(CH_3)-NH$ (Cl), HCl

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La synthèse marquée du sulfate de (±)-amphétamine a été décrite par Wilson (1950) en partant de l'acide phénylacétique ^{14}C-2 et par Blackburn *et al.* (1965) à partir de l'acide benzoïque ^{14}C-7 par l'intermédiaire du chlorure de benzoyle et du benzaldéhyde. Ces derniers auteurs ont ensuite obtenu le sulfate de (+)-amphétamine *via* le (+)-tartrate dans des conditions très rigoureuses de température pour atteindre le meilleur rendement après plusieurs cristallisations. Ce procédé de séparation est le plus classique (Leithe, 1932; Temmler, 1939; Nabenhauer, 1942).

Nous avons synthétisé la (+)-amphétamine en suivant le schéma de Blackburn avec plusieurs modifications, notamment pour le dédoublement. A partir de $Ba^{14}CO_3$, nous avons préparé l'acide benzoïque ^{14}C-7 suivant Baret *et al.* (1951), l'alcool benzylique ^{14}C-7 en solution pyridinique suivant Pichat *et al.* (1959) et le benzaldéhyde ^{14}C-7 en solution pyridinique suivant Herbert *et al.* (1969). Sans isoler l'aldéhyde, en condensant cette solution avec le nitroéthane en présence de benzène, nous avons obtenu le phényl-1 nitro-2 propène ^{14}C-1 que nous avons réduit en (±)-amphétamine ^{14}C-7 par $LiAlH_4$.

Par addition d'acide (+)-tartrique (1,1 mole pour 1 mole) au mélange en quantités égales de (±)-amphétamine ^{14}C-7 et de (+)-amphétamine non radioactive au sein du méthanol, nous avons préparé le (−)-tartrate de (+)-amphétamine ^{14}C-7. Après une seule cristallisation du (+)-tartrate dans l'isopropanol aqueux, la pureté optique est très satisfaisante et nous avons obtenu après alcalinisation, la (+)-amphétamine ^{14}C-7 avec une pureté optique de 97 % et un rendement de 72 %. A partir des eaux mères en utilisant le même procédé mais en diluant avec de la (−)-amphétamine non radioactive et en préparant le (−)-tartrate, on obtient la (−)-amphétamine ^{14}C-7.

Enfin, nous avons synthétisé le chlorhydrate de (+)-N-(o-chlorobenzyl) α-méthyl phénéthylamine en adaptant le procédé décrit par Boissier *et al.* (1966) aux conditions de la synthèse marquée.

Signalons que nous avons essayé sans succès plusieurs autres méthodes de séparation des antipodes optiques de la (±)-amphétamine : par l'intermédiaire des sels avec l'acide (−)-N-acétyl glutamique et le (−)-N-acétyltryptophane selon Gillingham (1955), par chromatographie sur couche mince d'alumine ou de silicagel imprégnée d'acides (+)-galacturonique, (+)-tartrique, (+)-mandélique ou (+)-quinique selon le procédé utilisé par Paris *et al.* (1967) dans le cas de l'éphédrine et enfin par chromatographie sur papier dans le système butanol-acide acétique-eau suivant Alessandro *et al.* (1954).

Partie expérimentale.

Acide benzoïque ^{14}C-7 (I).

On fait réagir H_2SO_4 concentré sur 1,96 g (10 mM) de $Ba^{14}CO_3$ (495 mCi) et on condense le $^{14}CO_2$ dégagé dans 25 ml d'une solution éthérée 0,45 M de

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bromure de phényl magnésium refroidie à −20 °C. Après hydrolyse, extraction et séchage, on obtient 0,923 g d'acide benzoïque ^{14}C-7 d'une activité de 370 mCi (Rendement en activité : 75 %).

Alcool benzylique ^{14}C-7 (II).

On dissout 0,923 g (7,6 mM) de (I) dans 25 ml d'éther anhydre. On réduit à l'aide de 100 ml d'une solution éthérée 0,22 M de $LiAlH_4$. Après hydrolyse, on extrait à l'éther. L'activité de l'alcool benzylique obtenu est de l'ordre de 352 mCi. On ajoute 50 ml de pyridine, on chasse l'éther à travers une colonne Vigreux et on obtient l'alcool benzylique en solution pyridinique (Rendement en activité : 95 %).

Benzaldehyde ^{14}C-7 (III).

On ajoute 7 g de $Pb(OAc)_4$ à la solution pyridinique d'alcool benzylique précédente et on porte le mélange à l'ébullition pendant 1 h sous agitation. On distille sous vide et on obtient une solution pyridinique de benzaldéhyde ^{14}C-7 d'une activité de l'ordre de 330 mCi exempte d'alcool benzylique par G.L.C. (Rendement en activité : 94 %).

Phényl-1 nitro-2 propène ^{14}C-1 (IV).

On ajoute 50 ml de benzène, 5 g (60 mM) de nitroéthane et 0,3 ml de pipéridine à la solution pyridinique de benzaldéhyde ^{14}C-7 (env. 6,8 mM). On porte à l'ébullition sous agitation pendant 24 h. La fin de la réaction est déterminée par disparition du benzaldéhyde (G.L.C.). On évapore le solvant sous pression réduite, on reprend le résidu huileux par l'éther et on lave plusieurs fois avec de l'acide chlorhydrique dilué, puis avec de l'eau. On sèche la solution éthérée sur sulfate de sodium anhydre. On obtient environ 320 mCi. L'éther est évaporé sous pression réduite et on obtient 1,07 g de produit qui s'identifie par spectrométrie U.V. dans l'alcool $\lambda_{max}(\varepsilon)$: 226 nm (10300); 305 nm (12400) (Rendement en activité : 97 %). La pureté radiochimique est de l'ordre de 85 %.

(±)-Amphétamine ^{14}C-7 (V).

On réduit 1,07 g (6,4 mM) de (IV) en solution éthérée avec 125 ml d'une solution éthérée 0,22 M de $LiAlH_4$. Après hydrolyse et extraction, on obtient une solution éthérée de (±)-amphétamine ^{14}C-7 d'une activité de l'ordre de 270 mCi (Rendement en activité : 84 %). La pureté radiochimique est de l'ordre de 85 %. Par chromatographie sur colonne de silicagel (éthanol/ammoniaque 4 %), on élimine en tête une impureté active (8 % de l'activité totale du produit) et on obtient la (±)-amphétamine ^{14}C-7 d'une pureté d'environ 93 %. On dissout la base dans le cyclohexane, on titre par spectrophotométrie U.V.

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et on ajoute la quantité théorique d'acide sulfurique 0,01 N avec un léger défaut (−2 %). On agite les deux phases durant une nuit, on décante la phase organique et on lave la solution aqueuse de sulfate de (±)-amphétamine à l'éther. On obtient une substance d'une pureté radiochimique de 98 à 99 %.

On alcalinise la solution aqueuse de sulfate de (±)-amphétamine ^{14}C-7 au moyen de 10 ml de NaOH (10 N) et on extrait à l'éther dans un extracteur liquide-liquide en continu. On chasse l'éther sous vide et on dissout le résidu huileux de (±)-amphétamine ^{14}C-7 dans 2 ml de méthanol. La concentration de cette solution est déterminée par dosage U.V., puis ajustée de manière à obtenir une solution 1 M.

Dédoublement optique : (±)-amphétamine ^{14}C-7 (VIa).

On utilise un ballon à deux cols rodé de 10 ml muni d'un réfrigérant à reflux, d'un compte gouttes et d'un verre fritté au fond du ballon. Le ballon est rendu étanche par application sur la surface externe du fritté d'un bouchon gainé d'une membrane en Téflon. On introduit dans le ballon 0,33 g (2,2 mM) d'acide (+)-tartrique en solution dans 3 ml de méthanol, puis goutte à goutte, à une température de 60 °C, une solution de 0,135 g (1 mM) de (±)-amphétamine ^{14}C-7 (49 mCi/mM) mélangée à 0,135 g (1 mM) de (+)-amphétamine inactive dans 1 ml de méthanol. On porte la solution à ébullition durant 1 min, puis on laisse revenir lentement à température ambiante et on abandonne pendant 16 h à 20 °C. On enlève le bouchon fermant le fritté, on essore le précipité et on sèche à 50 °C sous vide. On applique à nouveau le bouchon sur le fritté et on recristallise le produit à chaud dans env. 4,9 ml d'isopropanol aqueux (91 %). On laisse refroidir et reposer durant 4 h. On essore les cristaux selon la même technique que précédemment et on sèche sous vide à 50 °C. Dans le même ballon, on dissout les cristaux de (+)-tartrate de (+)-amphétamine ^{14}C-7 dans l'eau, on filtre, on alcalinise et on extrait la (+)-amphétamine à l'éther. Après évaporation lente de l'éther sous pression réduite, on obtient 0,145 g (1,1 mM) de (+)-amphétamine ^{14}C-7 (16,6 mCi). Le rendement, par rapport à la quantité totale d'isomères dextrogyres dans le mélange dédoublé, est de 72 % et la pureté optique du produit est de l'ordre de 97 %, déterminée par chromatographie en phase gazeuse. La pureté radiochimique du produit est supérieure à 99 %, déterminée par chromatographie sur couche mince.

Dédoublement optique : (−)-amphétamine ^{14}C-7 (VIb).

Les eaux-mères de cristallisation de plusieurs opérations de dédoublement sont réunies, alcalinisées et extraites à l'éther. On récupère 275 mg (2 mM) d'amphétamine dont l'analyse par G.L.C. montre que le produit est pratiquement racémique. On ajoute 275 mg de (−)-amphétamine non radioactive dissoute dans 3 ml de méthanol. On ajoute 660 mg d'acide (−)-tartrique et on procède au dédoublement selon la même technique que celle décrite pour la (+)-amphétamine. On obtient 275 mg de (−)-amphétamine ^{14}C- d'une activité spécifique de 15 mCi/mM.

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Chlorhydrate de (+)-N-(o-chlorobenzyl) α-méthyl phénéthylamine ^{14}C-7 (VII).

Dans un ballon rodé à deux cols de 5 ml muni d'un refrigérant à reflux, d'une entrée d'azote et d'une agitation magnétique, on introduit 0,11 g (0,815 mM) de (+)-amphétamine ^{14}C-7 (12,5 mCi) en solution dans 1,1 ml de méthanol et 0,11 g (0,785 mM) d'o-chlorobenzaldéhyde. On agite à la température ambiante pendant 16 h sous azote. On ajoute en agitant 0,04 g de borohydrure de sodium par petites portions. On agite pendant 1 h à la température ambiante, puis on chauffe pendant 2 h à reflux. On refroidit et on ajoute goutte à goute 2 ml d'eau. On obtient une suspension, qui est transvasée par pipettage dans un ballon à fond fritté du type décrit précédemment. On ajoute 0,5 ml d'acide chlorhydrique concentré et on laisse reposer pendant 2 h. On essore le précipité, on le lave avec deux fois 0,5 ml d'eau et on sèche à 50 °C sous vide. On obtient 0,145 g de (VII) (7,6 mCi), identifié par spectre U.V. dans le méthanol λ_{max} (ε) : 273 nm (266); 278 nm (413). Le rendement est de 61 % par rapport à la (+)-amphétamine ^{14}C-7. L'activité spécifique du produit est de 17,2 mCi/mM ou 58,1 μCi/mg. La pureté radiochimique du produit final a été déterminée par chromatographie sur papier. Elle est de l'ordre de 99 %.

DONNÉES ANALYTIQUES.

Chromatographie en phase gazeuse (G.L.C.)

Colonne de 2 m de Silicone OVI sur Chromosorb W; détecteur à ionisation de flamme; température d'injection : 300 °C.
Temps de rétention :

. Benzaldéhyde : 3.62 mn. colonne à 105 °C
. Alcool benzylique : 5.78 mn. colonne à 105 °C
. Phényl nitropropène : 2.05 mn. colonne à 205 °C

Détermination de la pureté optique de la (+)-amphétamine :

Méthode de Gordis (1966); réaction avec la trifluoroacétyl-1 chlorocarbonyl-2 pyrrolidine (TCP) et G.L.C.
Colonne en verre de 2 m, NPGS 3 % sur Chromosorb W; détecteur à ionisation de flamme; température d'injection : 270 °C.
Temps de rétention :

. (−) TCP-(−)-amphétamine : 30.53 mn. colonne à 270 °C
. (−) TCP-(+)-amphétamine : 34.71 mn. colonne à 270 °C

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Chromatographie sur couche mince de silice de la (+)-amphétamine :

Système	*Rf*
Chloroforme/méthanol 5/5	0.28
Chloroforme/méthanol/acide acétique 7.5/2/5	0.85
Méthyléthylcétone/diméthylformamide/ammoniaque 13/1.9/0.1	0.78
Butanol/acétone/eau 8/1/1	0.25
Ethanol/ammoniaque 9.8/0.2	0.35
Butanol/acide formique/eau 7.5/1.5/1	0.75

Chromatographie du chlorhydrate de (+)-N-(o-chlorobenzyl) α-méthyl phénéthylamine ^{14}C-7 :

a) — *sur papier*

Système	*Rf*
n-butanol/pyridine/sol. saturée NaCl/eau 40/80/60/10.5	0.91
toluène/n-butanol/ac. acétique/eau 2/2/1/1	0.82
n-butanol/NaOH 1,5 N 1/1	0.91

b) — *sur couche mince*

Système	*Rf*
toluène/butanol/acide acétique/eau 2/2/1/1	
— sur cellulose, support aluminium	0.91
— sur silice, support aluminium	0.74

* * *

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ETUDE PAR SPECTROSCOPIE RAMAN DE QUELQUES ANOREXIGENES—I

ANOREXIGENES *N*-SUBSTITUES

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Résumé—Les spectres Raman complets de trois anorexigènes ont été enregistrés à l'état polycristallin. Une attribution des raies observées est proposée. Elle montre un certain nombre de similitudes dues essentiellement à la structure phénylalkylaminique de ces composés dont les raies caractéristiques apparaissent à 3060, 1600, 1205, 1157, 1028, 1000, 995, 618 et 460 cm^{-1}. Cependant certaines différences apparaissent sur les spectres, il a été noté en particulier que les raies situées à 3160, 3120 (et l'absence de raie à 3092 cm^{-1}), 1500, 1393 et 802 cm^{-1} sont spécifiques du cyclohexylsulfamate de furfenorex et que les raies situées à 682 et 587 cm^{-1} et 2257 cm^{-1} caractérisent respectivement la chlorhydrate de clobenzorex et le diphénylacétate de fenproporex.

Nous présentons dans ce travail les spectres Raman de trois dérivés d'une famille de composés non phénoliques de structure phénylalkylaminique (du type de l'éphédrine) dans laquelle on trouve:

— d'une part les amines de réveil et d'autres stimulants du système nerveux central,

— d'autre part les anorexigènes qui nous intéressent ici plus particulièrement.

Dans ce premier volet nous présentons et discutons les spectres Raman de trois composés, dérivant de l'amphétamine par différentes substitutions sur l'atome d'azote, et stabilisés sous forme de sels: le cyclohexylsulfamate de furfenorex; le chlorhydrate de clobenzorex; le diphényl acétate de fenproporex (tableau 1).

PARTIE EXPERIMENTALE

Appareillage

Les spectres Raman ont été enregistrés sur un spectromètre Coderg T 800 comprenant un triple monochromateur de 800 mm de distance focale équipé de trois réseaux plans gravés (1800 traits par mm).

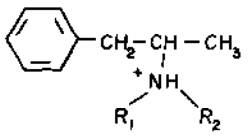
Un laser à argon ionisé (514,5 nm) Spectra Physics modèle 165,03 délivre 600 mW sur l'échantillon. Les principes actifs sont introduits sous forme de poudre polycristalline dans un tube capillaire sans purification supplémentaire.

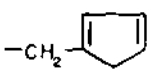
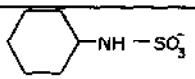
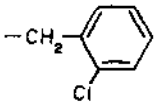
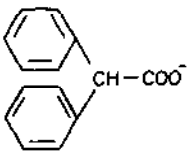
Réactifs

Les principes actifs dont nous avons disposés sont destinés à la fabrication de comprimés ou de gélules. Leur pureté a été contrôlée à l'issue de leur synthèse par des techniques classiques telles que la spectrométrie infra

Tableau 1. Structure des anorexigènes étudiés

Structure générale



	Composé	R_1	R_2	X^-
I	Cyclohexyl sulfamate de furfenorex	$-CH_3$		
II	Chlorhydrate de clobenzorex	$-H$		Cl^-
III	Diphényl acetate de fenproporex	$-H$	$-CH_2-CH_2-CN$	

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rouge, la résonance magnétique nucléaire, la chromatographie en phase gazeuse, la potentiométrie en milieu non aqueux, dont le détail sort du cadre de ce travail. Signalons à titre d'exemple que le protocole de contrôle du diphénylacétate de fenproporex fait l'objet d'un projet qui sera bientôt publié dans la Pharmacopée Française.

Methode

Nous donnons à titre indicatif les raies principales du spectre Raman de l'amphétamine base dans le tableau 2. Cette amine est instable et se volatilise légèrement à température ambiante, c'est pourquoi son spectre a été enregistré en figeant la substance en la refroidissant à −80°C.

Les valeurs moyennes en cm^{-1} dans le tableau 2 sont obtenues à partir de deux enregistrements par échantillon.

Les spectres ont été enregistrés à la vitesse de 25 cm^{-1}/mn avec une résolution spectrale de 2 cm^{-1}. Cette vitesse a été choisie de façon à pointer les raies avec la meilleure précison. Elle peut être augmentée lors d'étude de routine pour devenir comparable à celle couramment employée en spectrométrie infra rouge.

Bien que les deux types de spectrométrie IR et Raman soient complémentaires, la spectrométrie Raman présente l'avantage de minimiser les manipulations lors de la préparation de l'échantillon. Il suffit en effet dans la plupart des cas d'introduire quelques milligrammes de produit dans un tube capillaire en verre. D'autre part le spectromètre Raman utilisé permet d'enregistrer en continue le spectre d'un composé de quelques cm^{-1} à 4000 cm^{-1}. Cependant les problèmes parfois rencontrés dans ce type de spectroscopie sont essentiellement dus à la fluorescence extrinsèque de l'échantillon, fluorescence qui peut très souvent être inhibée par irradiation sous le faisceau laser. Parfois certaines molécules ne résistent pas à la puissance du rayonnement et se détruisent au niveau de l'impact du faisceau sur le tube capillaire.

L'enregistrement est alors possible

— soit en diminuant la puissance du laser, l'intensité des raies décroissant proportionnellement,

— soit en n'exposant qu'une surface donnée de l'échantillon pendant un temps très court en utilisant une cellule tournante ou le dispositif d'irradiation laser (D.I.L.) mis au point par notre groupe.

Lors de l'enregistrement des spectres des anorexigènes cités dans ce travail nous n'avons pas rencontré ce type de problème.

RESULTATS ET DISCUSSION

Fréquences caractéristiques dans les spectres Raman

Les substances étudiées présentent en commun la structure phénylalkylaminique. Les différences apparaissent au niveau de la substitution sur l'atome d'azote salifié par différents acides (*cf.* tableau 1). Il est donc logique de penser qu'un certain nombre de raies apparaîtrent sur les spectres à des fréquences voisines. Nous étudierons donc les raies des groupements communs dans un premier temps. Cependant l'intérêt de ce travail réside aussi dans l'étude des raies spécifiques qui permettrent de différencier les trois composés.

Les resultats sont rassemblés dans le tableau 2 où figurent les fréquences observées dans les spectres Raman qui ont servi de base à l'attribution. Nous donnons également les fréquences IR des divers composés.

A titre d'exemple les figures 1 et 2 représentent respectivement les spectres Raman et IR du chlorhydrate de clobenzorex.

La nomenclature adoptée pour les modes de vibration du noyau benzénique est celle de Wilson.[1]

Raies dues aux groupements communs

Cycle benzénique monosubstitué. Pour le cycle phényl monosubstitué 24 vibrations sont essentiellement indépendantes du substituant attaché au noyau, les 6 autres sont sensibles aux substitutions puisque dans ces modes le substituant vibre avec une amplitude appréciable.[2]

Il y a 5 vibrations de valence des CH dont les fréquences tombent dans l'intervalle 3100–3000 cm^{-1}. Goubeau et Kohler indiquent que la fréquence moyenne de la bande Raman dans les monoalkylbenzènes sur les vibrations du CH aromatique est à 3057 cm^{-1}.[3]

Tableau 2.

Cyclohexyl sulfamate de furfenorex (I)				Chlorhydrate de clobenzorex (II)				Diphenyl acetate de fenproporex (III)				
Raman		Infra rouge		Raman		Infra rouge		Raman		Infra rouge		
ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	Essai d'attribution
3160	mF							3160	ff			3160 bandes caractéristiques de la substitution en 2 sur le noyau furannique
3120	mF											3120
						3088	ep					
		3078	ep							3082	ep	
3069	ep			*3073*	ep							
3064	ep											
				3063	F							3057 ν(CH) aromatiques
								3061	F			3063
										3059	ep	3061 + les épaulements
3057	F											
						3055	ep					
								3049	ep			
3048	ep											
				3044	f							

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Tableau 2—*suite*

Cyclohexyl sulfamate de furfenorex (I)				Chlorhydrate de clobenzorex (II)				Diphenyl acetate de fenproporex (III)				
Raman		Infra rouge		Raman		Infra rouge		Raman		Infra rouge		
ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	Essai d'attribution
				3035	f							
								3033	ep			
						3031	ep					
										3026	f	
				3018	f	3018	ep					
3005	f											
								3002	ff			
				2998	f							
				2983	f	2983	ep					
								2982	ff			
										2972	ep	
2965	F											2965 νCH_3 dégénérée
								2952	f			
2937	F											2937
								2936	ep			2932 ν_{as} CH_2
						2933	F					2931
				2932	mF							
								2931	mF			
		2923	F							2930	ff	
								2907	ff			
2903	ep											
2891	ep											
2884	ep											
				2865	f							
2848	F											
		2836	ep									
						2832	ep					
		2812	ep									
						2792	F					
				2790	ff							
				2759	ff							
		2757	ep							2757	f	
		2710	ep									
						2700	ep					
		2650	ep							2670	f	
		2596	ep									
		2547	ep									
						2532	mF					
		2489	ep							2442	f	
						2440	ep					
						2402	mf					
						2365	ep					
		2356										
								2257	f			2257 Groupement ν (C ≡ N)
										2250	f	
		2066	ep									
		2000	f									
										1965		
		1944	ff									
		1652	ep									
										1622	mf	
		1615	ep									
				1602	F			*1602*	F			1601, 1602, 1602 8a
1601	F											1586, 1584, 1583 8b
		1600	mf			1600	f					(1603 et 1596 amphétamine base)
										1597	ep	
						1595	f					
		1590	ep									
1586	ep											
				1584	ep							
								1583	f			
				1573	F							
						1570	ep					
						1577	mf					
		1544	mf									

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Tableau 2—*suite*

Cyclohexyl sulfamate de furfenorex (I)				Chlorhydrate de clobenzorex (II)				Diphenyl acetate de fenproporex (III)				
Raman		Infra rouge		Raman		Infra rouge		Raman		Infra rouge		
ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	Essai d'attribution
		1530	ep							1530	F	
						1508	ff					
1500	F											1500 élongation du cycle furfuryle substitué en 2
						1493	mf			1492	mf	
		1490	mf									
		1482	ep	1482	ff							
						1478	mf					
1461	ep											
		1460	f	1460	f							
						1459	ep					
										1450	mf	
				1447	mF							
		1445	mf									
								1444	ff			
1441	F											
		1440	ep			1439	F					
								1432	ff			
				1430	f							
						1429	ep					
										1424	ep	
						1412	e					
1393	mF											1393 élongation du cycle furannique seul
		1382	mf	1382	f	1382	mf					
								1372	ff			
										1366	F	
		1364	f									
				1355	mF							
						1349	ff					
1348	f											1348 vibration de déformation
												1341 du carbone tertiaire du
		1344	ff									1342 groupement isopropyle
								1342	ff			
				1341	f							
		1332	ff									
1331	f											
										1325	f	
		1314	ff									
				1310	ff							1307 b_{2u} élongation du cycle
												1310 aromatique confondue
1307	ff							*1306*	ff	1306	ep	1306 avec les vibrations de torsion et de balancement —CH
						1304	f					
				1285	mF							
								1284	ff			
										1280	ff	
						1279	f					
1276	ep											
		1275	f									
1266	mF											
								1260	ff			
										1254	ff	
		1253	f									
				1250	ff							
1240	f					1240	ff					
								1236	ff			
										1230	f	
1226	f											1226 vibration de déformation du CH dans le plan
		1223	ep									1209 vibration d'élongation
1209	F											1208 C_6H_5 C
				1208	F							

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Tableau 2– *suite*

Cyclohexyl sulfamate de furfenorex (J)				Chlorhydrate de clobenzorex (II)				Diphenyl acetate de fenproporex (III)				Essai d'attribution
Raman		Infra rouge		Raman		Infra rouge		Raman		Infra rouge		
ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	
						1204	ff			1202	ν_{ar-C}	
								1202	mF			(1206 amphétamine base)
								1195	ep			
1188	f											
										1187	ff	
				1181	mF							
		1175	ep					1175	mF			
						1174	ff					
				1168	f							
1157	mF							*1157*	mF	1165	ff	1157
				1155	mF							1155 CH déformation dans le
												1157 plan cycle aromatique
1153	ep											no 15
										1148	f	(1157 amphétamine base)
		1145	ep									
						1142	f					
1140	ep											
				1125	f							
		1120	F			1120	f					
										1108	ff	
								1091	ff			
1089	ep											
				1088	mF							
										1083	f	
						1081	mf					
								1077	ff			1076
1076	F											1088 déformation plane de
		1065	ep									1077 CH 18b
1064	ep									1062	f	
				1059	ep							
				1054	mF							
1051	ep					1051	F					
		1050	ep									
						1040	f					
				1037	F					1036	ep	1029 vibration des CH
												1037 aromatiques dans
				1031	ep			*1031*	F			1031 le plan 18a
1029	F											(1030 amphétamine base)
						1025	f					
										1023	f	
		1012	ep									
		1003	ep									
				1002	F							999 respiration trigonale
								1001	F			1002 du cycle b_{1u}
999	F					999	ep					1001 no 12
994	ep											(1000 amphétamine base)
991	ep							*990*	ep			991 déformation des CH
986	ep			986	ep							986 cycle aromatique
										982	f	990 vibration no 5
						980	mf					(988 amphétamine base)
		976	ep					970	ff			
		960	ep									
				957	ff							
956	f											
						950	f					
								948	ff			
										942	ff	
929	f											
				927	mF			927	ff			
						920	ff					
		918	ep									
913	f											
								908	ff			
				907	f							
										906	f	

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Tableau 2—*suite*

Cyclohexyl sulfamate de furfenorex (I)				Chlorhydrate de clobenzorex (II)				Diphenyl acetate de fenproporex (III)				Essai d'attribution
Raman		Infra rouge		Raman		Infra rouge		Raman		Infra rouge		
ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	
						905	f					
900	f											
				898	f							
						894	ff					
								893	ff			
887	f											
										883	f	
		882	f									
				877	mF							
865	mF											
						862	f					
855	ep											
										851	ff	
								848	f			
				841	mF							
		840	f									
830	F									834	ff	
						831	f					
								821	f			
				820	F							
819	ep											
						815	ep					
813	F											
										810	ff	
								808	ep			
802	F											802 vibration ν_s (C–C) ou respiration du cyclo-hexane
790	ep							790	f			
										773	ff	
760	f											
				758	ff							
						751	F					
743	f							743	f			
				742	mF							
						737	F					
										735	ep	
730	f									730	mf	
										729	ep	
				727	ff							
						720	ep					
				702	f							
										698	F	
						690	F					
										688	F	682 bande caractéristique du groupement ortho C_6H_4CX (X = Cl)
				682	F							
						672	ep					
648	mF											
								642	f			
										633	mf	
626	mF			*620*	mF							626 Déformation du cycle 620 aromatique non substitué 619 dérive de la vibration no 6b du benzène (615 amphétamine base)
								619	mF			
613	ep											
				612	f							
						610	ff					
		608	F							608	ff	
								607	ep			
605	f											
		593	ep			593	f					
				587	F							
582	f											
										572	ff	

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Tableau 2—*suite*

Cyclohexyl sulfamate de furfenorex (I)				Chlorhydrate de clobenzorex (II)				Diphenyl acetate de fenproporex (III)				Essai d'attribution
Raman		Infra rouge		Raman		Infra rouge		Raman		Infra rouge		
ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	ν, cm^{-1}	I	
								567	f			
						565	ff					
554	f											554 ν_{as} SO_2
		546	f									535 ν_s SO_2
			f									
535	f					535	ff					
				529	f							
513	f											
						508	f					
				502	f							
										495	f	
		469	ep			468	mf					
467	f			*467*	f							
								465	f			
				457	f	457	ep					
		453	ep									
444	f											
				440	F							
								439	f			
		436	f									
						435	ep					
						421	ep					
411	f											
										397	f	
		391	f									
				387	mF							
						385	f					
								380	f			
						367	ff					
		343	ep									
						339	f					
				336	f							
		325	f									
										324	f	
								322	f			
						312	ff					
301	mF											
						285	ep					
282	ep							282	mF			
										275	f	
273	ep											
				272	ep							
268	mF											
258	ep											
						254	f					
										250	f	
								248	F			
				242	F	242	f					
						223	f					
				222	F							
								192	ep			
								173	mF			
170	f											
				166	F							
120	ep											
				113	ep							
				103	F							vibrations du réseau cristallin
81	ep											
				64	F							
				31	mF							
24	F											
				22	F							
				13	F							

Abréviations: I = intensité; F = fort; mF = moyennement fort; f = faible; ff = très faible; ep = épaulement.

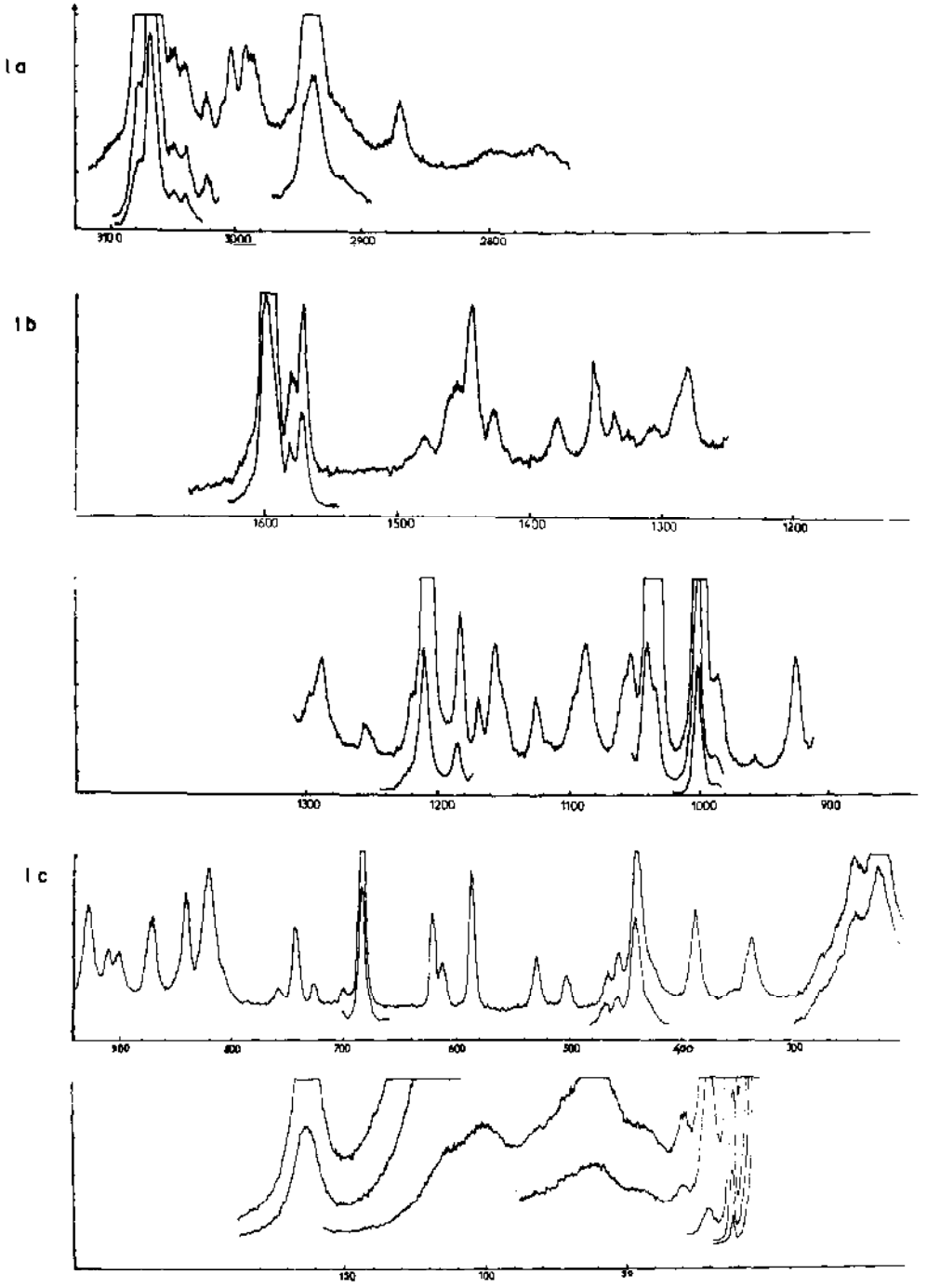

Fig. 1. Spectre Raman du chlorhydrate de clobenzorex: 1a—région spectrale 2700–3100 cm^{-1}; 1b—région spectrale 900–1600 cm^{-1}; 1c—région spectrale 0–950 cm^{-1}.

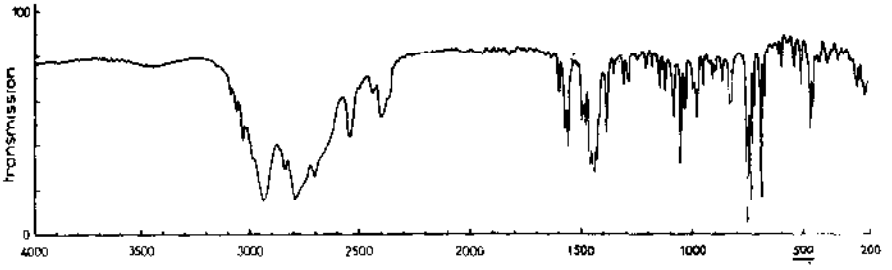

Fig. 2. Spectre IR (200–4000 cm^{-1}) du chlorhydrate de clobenzorex.

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Les spectres des composés I, II, III montrent une bande intense située respectivement à 3057, 3063, 3061 cm^{-1} ce qui est en accord avec les auteurs précédents. De part et d'autre de la raie principale apparaissent suivant les cas de 2 ou 3 épaulements.

La raie dégénérée e_{2g} (8a, 8b) correspondant à l'élongation du cycle n'est pas fortement perturbée par la substitution de sorte que les deux fréquences Raman résultantes sont très voisines de celle du benzène (1596 cm^{-1}). Ce couple de vibration indique la présence d'un noyau aromatique mais ne permet pas de distinguer le type de substitution. Sur les spectres les paires de raies correspondant aux vibrations du benzène 8a et 8b apparaissent respectivement à 1601 et 1586 cm^{-1} pour I, 1602 et 1584 cm^{-1} pour II, 1602 et 1583 cm^{-1} pour III. Les raies attribuées à 8a sont beaucoup plus intenses que les raies associées aux modes 8b.

La vibration dégénérée e_{1u} à 1486 cm^{-1} peut aussi éclater en deux bandes (19a, 19b) qui sont très proches de la fréquence primitive cependant ces bandes ont d'une part une très faible intensité en Raman et d'autre part sont souvent masquées par les vibrations de déformation antisymétriques des groupements méthyle attachés au noyau, nous n'avons donc pas remarqué ces bandes sur les spectres Raman.

La vibration b_{2u} (1310 cm^{-1}) d'élongation du cycle n°14 est seulement légèrement décalée par la substitution mais apparaît avec une intensité très faible dans l'intervalle de fréquence couvert par les vibrations de torsion et balancement des méthylènes des substituants alkyle. Sur les spectres la raie de faible intensité située respectivement pour les trois composés à 1307, 1310, 1306 cm^{-1} peut donc difficilement être attribuée seule à la vibration b_{2u}.

La vibration d'élongation symétrique du cycle monosubstitué correspondant (992 cm^{-1}) à la vibration n°1 a_{1g} du benzène est sensible aux substituants et selon Varsanyi[4] doit se trouver dans l'intervalle 1100-1060 cm^{-1} pour les substituants lourds (masse supérieure à 25) et dans l'intervalle 830–620 cm^{-1} pour les substituants légers (masse inférieure à 25). Nous n'avons pas pu sur nos spectres caractériser cette vibration.

Plus intéressante est la vibration correspondante à la déformation du cycle dans le plan ou respiration trigonale du cycle. Elle dérive de la vibration b_{1u} du benzène n°12 (1010 cm^{-1}) et donne lieu, pour les benzènes monosubstitués, à une intense bande Raman située dans l'intervalle 1010–990 cm^{-1}. Cette bande très caractéristique s'est manifestée sur les spectres des composés I, II, III respectivement à 999, 1002, 1001 cm^{-1}.

Pour les benzènes monosubstitués une autre vibration de déformation du cycle dérivée de la vibration du benzène n°6b (606 cm^{-1}) donne lieu à une bande d'intensité moyenne aux environs de 618 cm^{-1}. Les bandes qui apparaissent sur les spectres à 626, 620, 619 cm^{-1} correspondent à cette vibration du benzène.

La fréquence de vibration n°6a des benzènes monosubstitués est assez variable par conséquent il n'a pas été fait de tentative d'attribution.

Des vibrations de déformation des C–H dans le plan (n°3, 9a, 9b, 15, 18a, 18b) c'est celle qui dérive de la 18a qui est la plus utile pour l'identification, elle se traduit par une bande intense en Raman dans l'intervalle 1030–1018 cm^{-1}, de plus elle est caractéristique de la monosubstitution. Elle correspond sur les spectres des composés étudiés aux bandes à 1029, 1037, 1031 cm^{-1}. Bien que la bande située à 1037 cm^{-1} s'écarte sensiblement des autres, il est cependant logique de l'assimiler à bande de même nature du fait de la similitude des trois spectres dans cette zone surtout caractérisée par le voisinage de la vibration b_{1u} n°12 du benzène et par l'intensité forte de ces trois bandes.

La bande issue de la 18b devrait être comprise dans l'intervalle 1082–1065 cm^{-1} mais son intensité très faible aussi en Raman qu'en IR n'a pas permis de la situer.

La vibration e_{2g} dégénérée (n°9a, 9b) à 1178 cm^{-1} est perturbée et donnerait une bande spécifique d'intensité faible en Raman à 1180–1170 cm^{-1} (n°9a) et une bande non spécifique à 410–160 cm^{-1} (n°9b).

Ces bandes faibles n'ont pas été observées sur les spectres. Une bande d'intensité moyenne 1160–1150 cm^{-1} est due à la vibration n°15, elle se manifeste respectivement à 1157 (I et III) et 1155 cm^{-1} (II).

La vibration n°3 (1330–1250 cm^{-1}) est très faible et n'est pas apparue sur les enregistrements de manière significative. De même pour les vibration de déformation du cycle hors du plan: n°4 (700-680 cm^{-1}), n°16a (420–390 cm^{-1}).

Pour les vibrations de déformation des C–H hors du plan du cycle seule la vibration n°5 donne lieu en Raman à une bande suffisamment intense (1000–970 cm^{-1}) qui est caractérisée par un épaulement du côté des basses fréquences sur la bande correspondant à la vibration à 1000 cm^{-1} attribuée à la "respiration" trigonale du cycle. Ces épaulements se trouvent sur les spectres respectivement à 991 (I), 986 (II), 990 (III).

La substitution par un groupement alkyle peut être identifiée par une bande de moyenne ou de forte intensité au voisinage de 1205 cm^{-1}. Les trois composés étudiés présentent cette bande intense à 1209 (I), 1208 (II), 1202 (III) cm^{-1} correspondant certainement à la vibration d'élongation C_6H_5–C.

Groupement isopropyle. La caractérisation du groupement isopropyle est très délicate dans des spectres aussi complexes.

Le groupement isopropyle présente une vibration vers 1350–1330 cm^{-1} qui peut être associé à une vibration de déformation —CH du carbone tertiaire. Cette vibration est rencontrée à 1348 (I), 1341 (II), 1342 cm^{-1} (III). Enfin dans les zones 1175–1165 cm^{-1}, 1150–1140 cm^{-1} correspondant à la déformation de l'isopropyle et 960–950, 920, 835–795 cm^{-1}, 465–430 cm^{-1}, 370–255 cm^{-1} existent des raies correspondant probablement à cette attribution et très

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souvent masquée par les vibrations d'autres groupements.

Les vibrations d'élongation —C—H— des alcanes apparaissent dans l'intervalle 3000–2700 cm^{-1}. Ces bandes sont généralement intenses en Raman et ne présentent pas de caractères de spécificité dans l'optique de notre étude. La bande la plus intense [2937 (I), 2932 (II), 2931 cm^{-1} (III)] correspond certainement à la vibration d'élongation antisymétrique CH_2.

La bande observée vers 2965 cm^{-1} est attribuée à la vibration d'élongation dégénérée du groupement méthyle.

L'essai d'attribution de quelques raies correspondant à la structure phénylalkylaminique des composés étudiés montre combien les spectres sont marqués par la présence du cycle benzénique monosubstitué. Nous rappelons les raies caractéristiques les plus importantes:

bande intense vers 1000 cm^{-1} avec un épaulement près de 995 cm^{-1}
— une bande moyenne ou forte vers 1028 cm^{-1}
— une bande vers 618 cm^{-1}
— une bande moyenne vers 1157 cm^{-1}
— un doublet intense vers 1600 cm^{-1}
— la bande d'élongation des CH aromatiques près de 3060 cm^{-1}
— une bande vers 460 cm^{-1} caractéristiques des alcanes ramifiés
— une bande vers 1205 cm^{-1} correspondant à la vibration d'élongation C_6H_5—C

Le spectre de l'amphétamine base confirme d'une façon logique cette structure phénylalkyaminique puisque l'on retrouve:

— une bande intense à 1000 cm^{-1} avec son épaulement à 988 cm^{-1}
— une bande moyennement forte à 1030 cm^{-1}
— une bande d'intensité moyenne à 615 cm^{-1}
— une bande de faible intensité à 1157 cm^{-1}
— un doublet intense vers 1600 cm^{-1} (1603 et 1596 cm^{-1})
— une série de bandes entre 2700 et 3000 cm^{-1} dont la plus intense se situe à 2906 cm^{-1} (vibration d'élongation des alcanes)
— une bande moyennement forte à 1206 cm^{-1}.

Bien que les attributions précédentes permettent de confirmer la structure d'une partie du squelette de la molécule des trois anorexigènes, la connaissance des fréquences spécifiques à chacun d'entre eux peut être intéressante. C'est pourquoi la partie suivante sera consacrée à l'étude des raies caractéristiques de chacun des composés.

Raies caractéristiques de chaque composé

Cyclohexylsulfamate de furfénorex. La substitution d'un atome d'hydrogène de l'atome d'azote par un groupement furfuryle apporte à ce composé une particularité qui doit permettre de le différencier des autres anorexigènes.

En effet les premiers travaux sur les spectres Raman des noyaux furanniques substitués en 2, 2–5 et 2–3 furent conduits par Matsuno et Han[5] et par Bonino et Manzoni-Anside.[6] Ces auteurs avaient déjà établi que le groupement furfuryle pouvait être aisément distingué des groupements furfurylidène-2 et furoyle-2 sur la base de leur spectre Raman dans la région 1600–1460 cm^{-1}. De plus Sobolev *et al.*[7] dans une étude sur les alkyle furannes, a montré que le spectre dans la région des fréquences d'élongation de la liaison C—H varie suivant le nombre et le type de substituant.

Classiquement un cycle furannique présente trois bandes intenses à 3157, 3122 et 3092 cm^{-1}. Ces bandes sont retrouvées à 3160 et 3120 cm^{-1} dans le cas du cyclohexyl sulfamate de furfénorex avec un changement dans leur intensité relative ce qui est considéré comme une première caractéristique de la substitution en 2 (sur le noyau) à laquelle s'ajoute la disparition de la bande à 3092 cm^{-1}.

Parmi les bandes caractéristiques du cycle d'un groupement 2 furfuryle:

— celle qui apparaît vers 1605–1590 cm^{-1} n'est pas spécifique puisque confondue avec l'une des vibrations e_{2g} du benzène monosubstitué;
— la plus spécifique est la bande de forte intensité qui apparait à 1500 cm^{-1}.

Cette bande n'est pas retrouvé sur les spectres des dérivés (II) et (III), elle pourra donc être choisie préférentiellement pour toute étude de caractérisation. Les deux bandes résultent de l'élongation du cycle furfuryle substitué en 2.

La vibration à 1393 cm^{-1} est spécifique de l'élongation du cycle furannique seul.

Les vibrations de déformation des —CH— "dans le plan" et "hors du plan" sont difficiles à mettre en évidence sur les spectres puisque confondues avec d'autres raies.

Le groupement NH de l'ion cyclohexylsulfamate n'apparait pas comme très significatif. Dans les amines secondaires par exemple la bande NH se situe vers 750–700 cm^{-1} donc difficilement décelable.

La vibration δNH est en général masquée par δCH_2. A l'opposé le groupement cyclohexyle présente une caractéristique importante. La bande située à 802 cm^{-1} réputée pour être la plus significative résulte de la vibration symétrique C—C ou respiration du cycle (forme chaise). Cette bande intense n'apparait pas sur les spectres des autres anorexigènes.

Les autres vibrations de ce cycle ν_{as} CH_2 (2933–2915 cm^{-1}), ν_s CH_2 (2897–2852 cm^{-1}), δ_s CH_2 (1452 cm^{-1}) sont confondues avec d'autres raies sur l'enregistrement.

Enfin les vibrations du groupement SO_2, ν_{as} et ν_s, n'ont pas été détectées, cependant les deux bandes décrites à 555 et 517 cm^{-1} existent sur le spectre à 554 et 535 cm^{-1} et pourraient être attribuées à ces vibrations.

Il n'a pas été trouvé de raies caractéristiques des

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sels d'amine tertiaire. Les modes de vibration ν_{C-N^+} (821 cm^{-1}) et $\delta_{C_3-N^+}$ (467 cm^{-1}) correspondent aux bandes observées à 819 et 467 cm^{-1} mais d'intensité trop faible pour pouvoir être utilisés en vue d'une caractérisation de composé.

Chlorhydrate de clobenzorex. Le présence d'un benzénique disubstitué fixé à l'atome d'azote apporte à cette molécule une spécificité se traduisant sur le spectre par une bande intense à 682 cm^{-1} caractéristique de groupe ortho C_6H_4CX (X = Cl). Cette bande est généralement accompagnée d'une deuxième bande intense qui semble être celle située à 587 cm^{-1} et qui n'étant pas retrouvée sur les spectres des deux autres composés est spécifique du chlorhydrate de clobenzorex.

Les sels d'amine secondaire présentent un massif d'absorption intense large et complexe qui s'étend généralement entre 3000 et 2600 cm^{-1} donc peu utilisable pour la caractérisation.

Les raies caractéristiques du chlorhydrate de clobenzorex sont donc situées à 682 et 587 cm^{-1}.

Diphényl acétate de fenproporex. L'élément le plus caractéristique de ce composé est sans doute le groupement $—CH_2—CH_2—CN$ qui présente une vibration à 2257 cm^{-1}.

Région spectrale des basses fréquences (0–250 cm^{-1})

Les spectres Raman de basse fréquence (<250 cm^{-1}) sont très caractéristiques de chaque composé. Les bandes observées sont très intenses en particulier à basse température (−173°). Dans cette région spectrale on s'attend à observer généralement des raies dues aux vibrations de réseau cristallin (cas des échantillons sous forme de poudre polycristalline) en plus de raies intenses dues à des mouvements de torsion (rotation interne) et des mouvements du squelette de la molécule. Néanmoins l'attribution reste très difficile et nécessite l'emploi de techniques variées telles la spectroscopie d'absorption du rayonnement infra rouge lointain, l'étude de la polarisation des raies Raman observées sur un monocristal ainsi que le calcul *a priori* des fréquences de vibration du cristal. Un tel travail sera envisagé ultérieurement sur cette série de composés.

Conclusion

La structure phénylalkylaminique prédominante est confirmée par la position d'un nombre élevé de raies intenses communes aux anorexigènes étudiés et à l'amphétamine base dont ils dérivent. Cependant malgré la complexité des spectres des trois anorexigènes, il a été possible sur la base des données de spectroscopie Raman de caractériser chacun des composés par une ou plusieurs raies spécifiques. Le cyclohexyl sulfamate de furfenorex se distingue par les bandes situées à 3160 et 3120 cm^{-1} et par l'absence de raie à 3092 cm^{-1}. Ces faits sont caractéristiques de la substitution en 2 sur le noyau furannique. Par ailleurs la présence du groupement furfuryle est confirmée par la raie intense à 1500 cm^{-1} et la raie à 1393 cm^{-1} qui dérivent de l'élongation du cycle furannique. La raie intense à 802 cm^{-1}, réputée pour être la plus significative du cyclohexane, résulte de la vibration symétrique C—C ou respiration du cycle. Bien que les deux autres composés soient moins riches en raies spécifiques il est cependant possible de les différencier: le chlorhydrate de clobenzorex présente deux bandes intenses situées à 682 et 587 cm^{-1}, celles-ci traduisent la présence d'un groupe ortho C_6H_4CCl fixé à l'atome d'azote. Enfin le diphénylacétate de fenproporex se distingue par la raie à 2257 cm^{-1} spécifique du groupement $—CH_2—CH_2—CN$.

Remerciements—Cette étude a été effectuée dans le cadre du contrat de récherche avec l'INSERM CRL n°77-1-206. 3 que nous remercions pour le soutien apporté. Nous remercions également les Laboratoires Pharmaceutiques Diamant et Bottu qui nous ont fourni les substances étudiées.

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Summary—Raman spectra of a series of three anorexigenic drugs in the polycrystalline state have been recorded. Several spectral similarities have been observed and these are assigned to the basic phenylalkylamine structure of these compounds at the following positions: 3060, 1600, 1205, 1157, 1028, 1000, 995, 618 and 460 cm^{-1}. However, bands specific to each compound characterize the spectra. Furfenorex cyclohexyl sulphamate, for example, displays bands at 3160, 3120, 1500, 1393 and 802 cm^{-1}. The 2-substituted furan ring is characterized by absence of a band at 3092 cm^{-1}. Clobenzorex hydrochloride displays bands at 682 and 587 cm^{-1}, whereas fenproporex diphenylacetate possesses a specific band at 2257 cm^{-1}.

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(54) Título: NUEVAS COMPOSICION FARMACEUTICA DE LIBERACION CONTROLADA PARA UNA AMINA SIMPATICOMIMETICA EMPLEADA EN EL TRATAMIENTO DE LA OBESIDAD.

(54) Title: NOVEL CONTROLLED RELEASE PHARMACEUTICAL COMPOSITION FOR A SYMPATHICOMIMETIC AMINE FOR TREATING OBESITY.

(57) Resumen

La presente invención describe una composición farmacéutica sólida de liberación modificada, que comprende: un compuesto sólido farmacéuticamente activo derivado de una amina simpaticomimetica, particularmente Clobenzorex o una de sus sales farmacéuticamente aceptables y un o más agentes diluyente, modificador de liberación, antiadherente, lubricante y de recubrimiento.

(57) Abstract

The present invention describes a modified release solid pharmaceutical composition, which comprises: a pharmaceutically active solid compound resulting from a sympathicomimetic amine, particularly Clobenzorex or a pharmaceutically acceptable salt thereof and one or more diluting, release modifier, anti-adhesive, lubricant ad coating agents.

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NUEVA COMPOSICIÓN FARMACÉUTICA DE LIBERACIÓN CONTROLADA PARA UNA AMINA SIMPATICOMIMETICA EMPLEADA EN EL TRATAMIENTO DE LA OBESIDAD

CAMPO DE LA INVENCIÓN

Esta novedosa invención de aplicación en el campo del control de peso se refiere a una composición farmacéutica de un producto anorexigénico derivados de aminas simpaticomimeticas denominado Clobenzorex y sus sales, utilizado como agente para el control, prevención y tratamiento de la obesidad exógena que evita los posibles olvidos de la segunda toma, ya que este medicamento se recomienda tomar dos veces al día, una por la mañana antes del desayuno y por la tarde antes de la comida, por lo que con esta nueva formulación basta tomar una dosis por la mañana, ayudando con ello al cumplimiento del tratamiento terapéutico, la liberación del fármaco es controlado por componentes farmacéuticamente aceptables que cumplen con las leyes de difusión de solutos en solventes.

ANTECEDENTES

La obesidad es una enfermedad crónica, compleja, de etiología multifactorial, que afecta a un gran número de

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individuos en el mundo. Se caracteriza por el aumento de los depósitos de tejido graso, resultado del desequilibrio entre el ingreso y el gasto de energía, el mayor acceso a la comida con un alto contenido de grasas, al que se le suma el sedentarismo de la vida moderna. Desde 1985 hasta nuestros días la obesidad ha aumentado el 75%; las dos últimas décadas del siglo anterior son las que mostraron el mayor crecimiento de esta patología a nivel mundial. La obesidad está próxima a superar al cigarrillo como causa evitable de muerte. La OMS estima que a nivel mundial actualmente mil millones de personas tienen sobrepeso, de las cuales 300 millones son obesas ("La obesidad , responsable del incremento en la factura de los sistemas de salud en el mundo", Dr Xavier Formiguera, Revista Fama Atención, Sección Endocrinología, pagina 4-10, 2004), lo cual tiene considerables complicaciones cardiovasculares que presentan los individuos con sobrepeso y obesidad comprenderemos el gran impacto sanitario y económico que tiene esta patología en la salud pública. La OMS ante tal panorama ha planteado la necesidad de declarar como epidemia a la obesidad y resaltan los costos que esta enfermedad tiene para la salud. La obesidad desde un punto de vista de atención a la salud debe ser tratada como un problema de salud que requiere un algoritmo orientador

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para el tratamiento de la obesidad que incluye dieta, ejercicio, fármacos antiobesidad y cirugía.

La versatilidad de los objetivos fármaco terapéuticos posibilitará una amplitud de puntos de abordaje que por el momento se encuentra fuera del alcance de la dieta terapia y del ejercicio.

Desde las drogas que actúan disminuyendo el ingreso energético hasta las que promueven un aumento de gasto, existe una extensa graduación y multiplicidad de mecanismos de acción

La importancia del sobrepeso y la obesidad radica en su relación con múltiples patologías altamente prevalentes como el síndrome metabólico, la diabetes mellitus tipo 2, la hipertensión arterial, la enfermedad coronaria , la aterosclerosis, la litiasis biliar, problemas psicológicos y respiratorios (apnea de sueño), síndrome de ovarios poliquísticos, osteoartrosis, dislipidemias, cáncer de mama, endometrio, próstata y colon. El riesgo de morbimortalidad para dichas patologías se incrementa dos a tres veces en sujetos obesos en comparación con los que presentan un índice de masa corporal normal. Hoy se considera al exceso de grasa corporal, que corresponde a la identificación de la obesidad el elemento clínico patológico

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que define a esta enfermedad crónica como un serio problema individual y de salud pública.

Además, ya se conoce que no es el exceso de grasa en sí, si no la distribución de la grasa corporal la que se constituye en un fuerte indicador para el desarrollo de hipertensión arterial, problemas cardiovasculasculares y principalmente diabetes mellutus tipo 2, aceptándose que el índice de morbimortalidad es mayor cuando la obesidad tiene un predominio central, es decir, cuando el exceso de tejido adiposo se localiza en la región abdominal, sea intraabdominal, viceral o ometal.

Las relaciones entre la obesidad y los factores de riesgo cardiovascular secundario al exceso de grasa corporal se explica en parte a través de un conjunto de anormalidades que se conocen en la actualidad con el nombre se síndrome metabólico, que incluye a la hipertensión arterial, hipertrigliceridemia, disminución de lipoproteínas de alta densidad (HDL), alteración de glucemia (en ayunas o postpradial) y obesidad visceral (diagnosticada cuando la frecuencia abdominal es mayor de 88 cm en la mujer y de 102 cm en el hombre). La presencia de al menos tres de los signos mencionados es suficiente para diagnosticar la existencia de síndrome metabólico, según los criterios del tratamiento de

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Adulto III del plan Nacional de Expertos para la Educación sobre Colesterol del 2001 en los Estados Unidos de América (ATP III), que es indicativo de aumento del riesgo de enfermedad arterial coronaria prematura y daño macrovascular. El síndrome metabólico suele acompañarse de un incremento en las lipoproteínas de baja densidad (LDL), Específicamente del subtipo pequeño y denso, que son particularmente aterogénicas, y de resistencia a la insulina que por si misma es inductora de hiperinsulinemia. Estas manifestaciones, merecedoras de consideración fisiopatológica y clínica por constituir estados dismetabólicos incuestionables, no se encuentran incluidas entre los elementos del diagnostico del síndrome mencionado en la clasificación del ATP III .

Durante los últimos 5 años, ha habido una explosión del crecimiento de los mecanismos involucrados en el ingreso y el gasto de energía. Producto de estos nuevos crecimientos o de la investigación de la cual derivan, están generando nuevos fármacos que podrán ofrecer tratamientos más efectivos contra la obesidad, sin riesgos significativos para la salud.

Los fármacos que reducen el ingreso energético pueden clasificarse de diferente manera, entre los más importantes se encuentran las monoaminas centrales que reducen la ingesta, las cuales comprenden la norepinefrina, dopamina,

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histamina, entre otros; y los llamados agentes noradrenérgicos, los cuales comprenden Mazindol, Fenproporex, Efedrina, Fentermina, Anfrepramona, Fenilproponolamina y **Clobenzorex**.

AGENTES NORADRENÉRGICOS

Los agentes noradrenérgicos incluyen mazindol, fenproporex, efedrina, fentermina, anfepramona (dietilpropión), fenilpropanolamina y **clobenzorex**. Otros fármacos como dextroanfetamina, metanfetamina y fenmetrazina, por su potencial de abuso y efectos secundarios serios no están indicadas para el tratamiento de la obesidad.

Los noradrenérgicos actúan incrementando la liberación de catecolamina de las terminales nerviosas que principalmente finalizan sus conexiones en el núcleo parabentricular del hipotálamo. Todos ellos, con excepción del mazindol son derivados de la 2- feniletilamina (fenetilamina).

El **Clobenzorex** es un polvo cristalino blanco o casi blanco, soluble en agua dentro de un pH fisiológico.

En el estado de la técnica se encuentran diversas composiciones de **Clobenzorex**, empleadas en el tratamiento de la obesidad exógena, las cuales deben ser administrados dos veces al día, lo que implica que el fármaco tenga picos de

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absorción importantes en los usuarios lo que ocasiona usos no apropiados de este medicamento, sobre todo cuando lo emplean conductores de camiones ya sea transporte de personal o de carga o deportistas que desean aumentar su rendimiento.

Entre las composiciones farmacéuticas existentes se encuentra el medicamento denominado Asenlix fabricado por Sanofi Aventis, el cual contiene 30 mg de **Clobenzorex**, el cual se vende como un aorexigénico, auxiliar en el tratamiento de la obesidad exógena. Utilizado en el tratamiento asociado a la dieta en pacientes con obesidad y un índice de masa corporal (IMC) igual o superior a 30 kg/m², que no hayan respondido al régimen dietético apropiado solo. En cuanto a la reducción de peso, sólo se ha demostrado su eficacia a corto plazo.

OBJETO DE LA INVENCIÓN

El objeto de la invención es una composición farmacéutica de liberación modificada mediante componentes farmaceuticamente aceptables que controlan la disolución por leyes de difusión en la formulación de un medicamento usado en el control, prevención y tratamiento de la obesidad crónica.

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Un segundo objetivo de la presente invención es proporcionar un medicamento en forma de tabletas y/o cápsulas con componentes farmaceuticamente aceptables que controlan la liberación del fármaco mediante leyes de difusión y permeabilidad.

Es también objeto de la presente invención proporcionar una composición farmacéutica sólida de liberación controlada, que comprende Clobenzorex y su sales farmacéuticamente aceptables y uno o más excipientes farmacéuticamente aceptables que controlan la liberación.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

FIGURA 1. Muestra el perfil de disolución de la formulación de producto de referencia, Asenlix, en donde se puede apreciar la liberación del 100% de clobenzorex a los 60 minutos.

FIGURA 2. Muestra el perfil de disolución de la formulación, objeto de la presente invención, en donde se puede apreciar que la liberación de clobenzorex ocurre de manera paulatina.

DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

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La presente invención se relaciona con composiciones farmacéuticas sólidas que comprenden el uso de un compuesto anorexigenico, particularmente de **Clobenzorex** y sus sales farmacéuticamente aceptables para la elaboración de un medicamento útil en el control, prevención y tratamiento de la obesidad crónica, caracterizadas dichas composiciones porque comprenden un novedoso proceso de liberación controlada del fármaco ayudando con ello al apego al tratamiento, evitándose picos del fármaco en el plasma sanguíneo.

Existen varios productos reportados en el estado de la técnica que son de liberación inmediata especialmente en forma de cápsulas, pero debido a que este fármaco tiene una considerable variabilidad farmacocinética y a que presenta efectos secundarios importantes, entre los que se encuentran dolor de cabeza e insomnio, se hace indispensable un sistema para controlar la liberación del fármaco y con ello evitar concentraciones plasmáticas que presenten picos del fármaco ya que altas concentraciones del mismo en sangre se relacionan con los efectos secundarios reportados en la literatura.

El Clobenzorex es utilizado en el tratamiento control y prevención de la obesidad. Actualmente el Clobenzorex se

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administra preferentemente por vía oral en dos dosis de 30 a 60 mg por día, divididas antes del desayuno y de la comida.

Consecuentemente se desarrollo un medicamento en forma de tabletas y/o cápsulas con componentes farmaceuticamente aceptables que controlan la liberación del fármaco mediante leyes de difusión y permeabilidad.

La presente invención se refiere a una composición farmacéutica sólida de liberación modificada, de un principio activo anorexigénico que comprende:

a) Un compuesto sólido farmacéuticamente activo derivado de una amina simpaticomimetica, particularmente **Clobenzorex** o una de sus sales farmacéuticamente aceptables;

b) un o más agentes diluyente, modificador de liberación, antiadherente, lubricante y de recubrimiento.

En donde, el agente anorexigenico es **Clobenzorex**, Clorhidrato de Clobenzorex, o cualquiera de sus sales farmacéuticamente aceptables.

Los términos "compuesto farmaceuticamente activo" y "sales farmacéuticamente aceptables" se refieren a moléculas solubles en agua. Preferiblemente el compuesto farmaceuticamente es un compuesto hidrofílico, más preferiblemente anorexigénico y más preferiblemente Clorhidrato de **Clobenzorex**.

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El Clorhidrato de Clobenzorex (clorhidrato de (+)-N-(2 Clorobenzil)-α-metilfenetil -amino) es un principio activo que disminuye la ingesta de alimentos y en menor medida aumenta el metabolismo.

El agente diluyente es seleccionado del grupo que consiste en celulosa microcristalina, celulosa microcristalina silicificada, manitol, lactosa, entre otros farmaceuticamente aceptables.

El agente modificador de liberación es seleccionado del grupo que consiste de Hipromelosa 100M, Hipremelosa 4M, etil celulosa, propilmetil celulosa, derivados de metacrilato, Carbopol entre otros farmaceuticamente aceptables.

El agente antiadherente es seleccionado del grupo que consiste de estearato de magnesio, dióxido de silicio, ácido esteárico entre otros farmaceuticamente aceptables.

El agente lubricante es seleccionado del grupo que consiste de estearato de magnesio, politilenglicol, pruv entre otros farmaceuticamente aceptables.

El agente recubrimiento es seleccionado del grupo que consiste de Eudragit E 12.5, Opadry, Advantia Prime entre otros farmaceuticamente aceptables.

La relación preferida P/P entre el principio activo y los excipientes, es de 10 a 80 mg de Clobenzorex y de 10 a

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990 mg excipientes. La composición objeto de la presente invención puede ser en forma de Tabletas, Tabletas recubiertos, cápsulas de gelatina dura o blanda, gránulos, microesferas etc.

Preferentemente cuando se usa en forma de Tabletas, estas comprende entre 30 a 60 mg de Clobenzorex y excipientes, por dosis.

La invención también se relaciona con un proceso para preparar la composición farmacéutica objeto de la presente invención.

La invención también se relaciona con el uso de la composición farmacéutica mencionada para la elaboración de un medicamento utilizado en el tratamiento control y prevención de la obesidad.

A continuación se presentan ejemplos ilustrativos más no limitativos, de las composiciones objeto de la presente invención:

La siguiente tabla muestra los componentes de la composición objeto de la presente invención.

Componentes	**Porcentaje Form 2**	**Porcentaje Form 3**	**Porcentaje Form4**	**Porcentaje Form 5**
Clobenzorex o sus sales	23,3	24,0	26,1	30,0
Diluyente	49,0	50,5	54,9	63,1
Modificador de liberación	23,3	24,0	26,1	30,0
Antiadherente	0,5	0,5	0,5	0,6

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Lubricante	0,1	1,0	1,1	1,3
Recubrimiento	2,9	3,0	3,3	3,8

EJEMPLOS:

A continuación se describen algunos ejemplos de composiciones específicas de la formula general anterior.

Ejemplo 1:

Compuesto	Peso en mg
Clorhidrato de Clobenzorex	24,0
Celulosa Microcristalina Silicificada	57
Hipromelosa 100 M	15,0
Estearato de Magnesio	1,0
Eudragit E 12,5	3,0

La forma farmacéutica así obtenida presenta un perfil de liberación tal que se libera entre el 15 y 45 % de la dosis en los primeros 60 minutos, entre el 45 y 70 % de la dosis en los siguientes 180 minutos, entre el 60 y 85 % de la dosis en los 360 minutos y el resto en los siguientes 360 min.

Ejemplo 2:

Compuesto	p/p
Clorhidrato de Clobenzorex	24,0
Manitol	50,5
Hipromelosa 4 M	24,0
Dioxido de Silicio	0,5
Estearato de Magnesio	1,0
Opadry	3,0
Solvente (Se evapora)	20,0

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La forma farmacéutica obtenida presenta un perfil de liberación tal que se libera entre el 15 y 45 % de la dosis en los primeros 60 minutos, entre el 45 y 70 % de la dosis en los siguientes 180 minutos, entre el 60 y 85 % de la dosis en los 360 minutos y entre el 90 y 100% cumpliendose 720 min. Cumpliendo con el perfil de disolución y liberación del activo de acuerdo a lo establecido.

Para la elaboración de formas farmacéuitas sólidas de las novedosas formulaciones objeto de la presente invención se desarrollaron los procedimientos idóneos para cada forma, las cuales incluyen: Tabletas, Tabletas recubiertos, cápsulas de gelatina dura o blanda, gránulos, microesferas, entre otras formas orales.

A continuación se describe a modo ejemplo el proceso de fabricación de tabletas; siendo evidente para un experto en la materia el desarrollo de los procesos de fabricación de la demás formas farmacéuticas a partir de la descripción de el proceso que se describe a continuación y quc consiste en los siguientes pasos:

<u>Produción del Núcleo:</u>

Tamizar por malla 40, los siguientes polvos:

Clorhidrato de Clobenzorex

Celulosa Microcristalina Slilicificada

Hipromelosa 4 M

Dioxido de Silicio

Adicionar a un mezclador de Pantalón la cantidad total de Clorhidrato Clobenzorex (27,00 g), así como el total de Hipromelosa 4 M (27,00 g), más la cantidad total de Dioxido de Silicio (0,56 g)

Mezclar durante 20 minutos los polvos en el mezclador de pantalón.

Adicionar 55 g de Celulosa Microcristalina Silicificada al mezclador de Pantalón y mezclar durante 25 min.

Tamizar por malla 40 el resto de la Celulosa Microcristalina Silicificada (1,81 g) junto con el total de Estearato de Magnesio (1,12 g) y adicionar al mezclador de pantalón. Mezclar durante 10 min.

Comprimir en una tableteadora Stokes con punzones cóncavos 8 mm.

Recubrimiento del Núcleo:

En un recipiente de acero inoxidable dispersar 3,38 g de Advantia Prime en 22,6 g de Agua Purificada. Agitar 60 minutos a una velocidad de 250 rpm.

Una vez alcanzada la temperatura requerida iniciar la aspersión del material de recubrimiento a una velocidad de

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flujo de aproximadamente 5,0 g/min. Rotar el sistema de laqueado, con una velocidad del bombo de aproximadamente de 19 rpm.

Mantener las condiciones de 37,5°C en los núcleos ajustando la temperatura en el termostato.

Una vez terminada la solución para laquear apagar la bomba y colocar la temperatura del termostato de aire a 25°C, mantener el bombo girando durante 30 minutos a 5 rmp para enfriar los núcleos.

Las composiciones obtenidas se analizaron mediante el método analítico desarrollado y que se describe a continuación:

Análisis Químicos para la Valoración:

Preparación de Fase móvil.

Solución fosfato de potasio monobásico 1,0 M

Pesar 136,1 g de fosfato de potasio monobásico, transferir a un matraz volumétrico de 1,000 ml, adicionar de 500 a 800 ml de agua purificada, disolver con agitación magnética, aforar a volumen con agua purificada. Guardar en frasco ámbar y en refrigeración.

Solución nitrato de sodio 1,0 M

Pesar 85,0 g de nitrato de sodio, transferir a un matraz volumétrico de 1 000 ml, adicionar de 500 a 800 ml de agua

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purificada, disolver con agitación magnética, aforar a volumen con agua purificada. Guardar en frasco ámbar y en refrigeración.

Fase móvil, proporciones para preparar 1,000 ml de fase móvil

Reactivo	Volumen
Agua	420
Fosfato de	60
Nitrato de	20
Acetonitrilo	500

Mezclar y ajustar el pH potenciométricamente a 3,0 con ácido fosfórico concentrado o con hidróxido de sodio 0,2 M filtre a través de una membrana de 0,45 micras, degasificar durante 15 minutos en sonicador.

Ácido clorhídrico 0,1 N (pH 1,2)

Adicionar aproximadamente 9,0 ml de ácido clorhídrico 10 N en un vaso de precipitados que contenga aproximadamente 1,000 ml de agua purificada y mezclar con agitación magnética. Medir el pH con un potenciómetro previamente calibrado, si es necesario adicionar más ácido clorhídrico hasta obtener una solución con un pH de 1,2, en caso contrario, ajustar el pH agregando agua purificada.

Preparación del estándar.

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Pesar con exactitud el equivalente a 15,0 mg del estándar secundario de Clorhidrato de **Clobenzorex** en un matraz volumétrico de 25 ml, disolver y bajar (enjuagar) con aproximadamente 15 ml de ácido clorhídrico (pH 1,2) sonicar durante 5 minutos. Mezclar y aforar con ácido clorhídrico pH 1,2.

Transferir una alícuota de 5 ml de la solución anterior a un matraz de 25 ml, aforar con fase móvil. Esta solución tiene una concentración final de 0,12 mg/ml. Filtrar la solución a través de una membrana de 0,45 micras.

Preparación de la muestra.

Retirar la cubierta de al menos de 20 tabletas de las tabletas obtenidas de 60 mg, moler, homogenizar y pesar por triplicado la cantidad de polvo equivalente a 60,0 mg. de Clorhidrato de **Clobenzorex**, transferir a un matraz volumétrico de 100 ml, bajar (enjuagar) la muestra con aproximadamente 50 ml de ácido clorhídrico (pH 1,2), agitar magnéticamente durante 15 minutos sonicar por 5 minutos, mezclar y aforar a volumen con ácido clorhídrico pH 1,2, filtrar la solución con papel filtro Whatman del No. 4 o equivalente, desechar los primeros 20 ml del filtrado.

Transferir una alícuota de 5 ml de la solución anterior a un matraz volumétrico de 25 ml mezclar y aforar a volumen

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con fase móvil. Esta solución tiene una concentración final de 0,12 mg/ml. Filtrar la solución por membrana de 0,45 micras.

Sistema Cromatográfico.

El cromatógrafo esta equipado con detector U.V. a 264 nm. Columna Symmetry C18 de 4,6 x 250 mm y 5 micras de tamaño de partícula. Velocidad de flujo de 1,5 ml/min. Volumen de inyección de 20 microlitros.

Verificación del sistema.

Inyectar un blanco de la fase móvil, determinar la verificación del sistema inyectando 20 µL de la solución estándar por sextuplicado.

Criterios de aceptación para la verificación del sistema

Por ciento de Desviación estándar relativa (%DER): no mayor del 2,0 por ciento (áreas)

Platos teóricos (N): No menos de 2 000

Factor de coleo (T): No mayor de 2,0

Procedimiento.

Una vez realizada la verificación del sistema inyectar 20 microlitros de la solución del estándar por triplicado y las muestras individuales.

El resultado de la concentración de Clorhidrato de **Clobenzorex** es calculado por el programa de integración del

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equipo, el cual imprime el resultado de las concentraciones y los cromatogramas.

Liberación del Principio Activo.

Determinar la cantidad de Clorhidrato de **Clobenzorex** 60 mg disuelto de acuerdo a la siguiente tabla.

TIEMPO	ESPECIFICACION
1,0 h (60 min.)	No menos de 20,0 por ciento y no mas de 40,0 por ciento (12,0 mg a 24,0 mg) de Clorhidrato de **Clobenzorex.**
2,0 h (120 min.)	No menos de 30,0 por ciento y no mas de 50,0 por ciento (18,0 mg a 30,0 mg) de Clorhidrato de **Clobenzorex.**
5,0 h (300 min.)	No menos de 60,0 por ciento y no mas de 80,0 por ciento (36,0 mg a 48,0 mg) de Clorhidrato de **Clobenzorex.**
8,0 h (480 min.)	No menos de 75,0 por ciento y no mas de 95,0 por ciento (45,0 mg a 57,0 mg) de Clorhidrato de **Clobenzorex.**
12,0 h (720 min.)	No menos de 90,0 por ciento y no mas de 115,00 por ciento (54,0 mg a 69,0 mg) de Clorhidrato de **Clobenzorex.**

Sistema de Disolución

Aparato: 2 (paletas)

Velocidad: 50 rpm

Medio de disolución: 1,000 ml agua purificada

Temperatura: 37 °C ± 0.5 °C

Tiempo: 720 min.

Celda: 0,1 cm.

Long. de Onda: 210 nm.

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Preparación del estándar.

Pesar el equivalente a 30,0 mg. del estándar de Clorhidrato de **Clobenzorex**, transferir a un matraz volumétrico de 50 ml, bajar (enjuagar) con aproximadamente 20 ml de agua purificada, sonicar 5 min., aforar a volumen con agua purificada.

Tomar las siguientes alícuotas independientes (3, 6, 8, 10 y 12 mL) y transferir cada una a un matraz volumétrico de 100 ml, aforar cada una con agua purificada y agitar. Las soluciones (curva de calibración) quedan 30, 60, 80, 100, y 120 por ciento y 0,018, 0,036, 0,048, 0,060, 0,072 mg/ml.

Procedimiento.

Para acondicionar el equipo es necesario que la distancia entre el agitador y el fondo del vaso se encuentre a 2,5 cm. + 2,0 mm.

Realizar la curva de calibración en el espectrofotómetro usando la longitud de onda de 210 nm y celda de cuarzo de 0,1cm. de paso óptico, utilizando como blanco agua purificada. Leer cada punto por triplicado.

Antes de adicionar a los vasos el medio de disolución, degasificar el medio calentando con agitación magnética y luego sonicando durante 15 minutos y proceder la

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dosificación, dejando caer lentamente y sobre las paredes de cada uno de los vasos para evitar burbujas.

Verificar que el equipo de disolución este en las condiciones especificadas para determinar el análisis.

Cuando se ejecute el método de corrida, adicionar una tableta de **Clobenzorex** de 60 mg en cada uno de los vasos cuando lo indique el programa de corrida. El programa automáticamente tomara las alícuotas necesarias y en el tiempo especificado, para la determinación.

El resultado de la concentración de Clorhidrato de **Clobenzorex** de las muestras es calculado por el programa del equipo, el cual imprime el resultado de la concentración, la grafica con el comportamiento y los datos del perfil de disolución. Finalmente los promedios de cada tiempo de la disolución, ilustrados en la figura 3.

El uso del medicamento objeto de la presente invención representa enormes ventajas para el paciente como son:

- Mejor apego a tratamiento
- Disminución de efectos secundarios como son dolor de cabeza e insomnio
- Uso mas apropiado del medicamento
- Mayor aceptación para pacientes con problemas de deglución

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REIVINDICACIONES

Habiéndose descrito la invención como antecedente, se reclama como propiedad lo contenido en las siguientes reivindicaciones:

1. Una composición farmacéutica sólida de liberación controlada, caracterizada porque comprende un compuesto sólido farmacéuticamente activo derivado de una amina simpaticomimetica, uno o más agentes diluyente, uno o más agentes modificador de liberación, uno o más agentes antiadherente, uno o más agentes lubricante y uno o más agentes de recubrimiento.
2. La composición de conformidad con la reivindicación 1, caracterizada porque la amino simpaticomimetica es **Clobenzorex** o cualquiera de sus sales farmaceuticamente aceptables.
3. La composición de conformidad con la reivindicación 1, caracterizada porque la amino simpaticomimetica es Clorhidrato de **Clobenzorex.**
4. La composición de conformidad con la reivindicación 1, caracterizada porque el agente diluyente es seleccionado del grupo que consiste en celulosa microcristalina, celulosa microcristalina silicificada, manitol, lactosa,

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entre otros farmaceuticamente aceptables; el agente modificador de liberación es seleccionado del grupo que consiste de Hipromelosa 100M, Hipremelosa 4M, etil celulosa, propilmetil celulosa, derivados de metacrilato, Carbopol, entre otros farmaceuticamente aceptables; el agente antiadherente es seleccionado del grupo que consiste de estearato de magnesio, dióxido de silicio, ácido esteárico, entre otros farmaceuticamente aceptables; el agente lubricante es seleccionado del grupo que consiste de estearato de magnesio, politilenglicol, pruv, entre otros farmaceuticamente aceptables; el agente recubrimiento es seleccionado del grupo que consiste de Eudragit E 12.5, Opadry, Advantia Prime, entre otros farmaceuticamente aceptables.

5. La composición de conformidad con la reivindicación 1, caracterizada porque la relación preferida P/P entre la amina simpaticomimetica, y los agentes diluyente, modificador de liberación, antiadherente, lubricante y de recubrimiento, es de 10 a 80 mg de la amina simpaticomimetica y de 10 a 990 mg de los agentes diluyente, modificador de liberación, antiadherente, lubricante y de recubrimiento.

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6. La composición de conformidad con la reivindicación 1, caracterizada porque se puede obtener en forma de Tabletas, Tabletas recubiertos, cápsulas de gelatina dura o blanda, gránulos, microesferas, entre otras formas sólidas.

7. La composición de conformidad con la reivindicación 1, caracterizada porque se puede obtener en forma de Tabletas.

8. Un proceso para la fabricación de tabletas que contienen la composición según cualquiera de las reivindicaciones precedentes, caracterizado porque el proceso comprende las siguientes etapas:

A) Producción del Núcleo; y

B) Recubrimiento del Núcleo.

9. El proceso de la reivindicación 8, caracterizado porque la producción del núcleo comprende los siguientes pasos:

a) tamizar en un Granulador por malla 40, la amina simpaticomimetica, uno o más agentes diluyente, uno o más agentes modificador de liberación, uno o más agentes antiadherentes, uno o más agentes lubricante y uno o más agentes de recubrimiento;

b) adicionar a un mezclador de Pantalón la cantidad total de la amina simpaticomimetica, así como el total de uno

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o más agentes modificador de liberación, más la cantidad total de uno o más agentes lubricante;

c) mezclar durante 20 minutos los polvos en el mezclador de pantalón;

d) adicionar uno o más agentes diluyente al mezclador de Pantalón y mezclar durante 25 min;

e) tamizar por malla 40 el resto de uno o más agentes diluyente junto con el total de uno o más agentes lubricante y adicionar al mezclador de pantalón; mezclar durante 10 min; y

f) comprimir en una tableteadora Stokes con punzones cóncavos.

10. El proceso de la reivindicación 8, caracterizado porque el recubrimiento del núcleo comprende los siguientes pasos:

a) dispersar en un recipiente de acero inoxidable uno o más agentes de recubrimiento en Agua Purificada. Agitar 60 minutos a una velocidad de 250 rpm;

b) una vez alcanzada la temperatura requerida iniciar la aspersión del material de recubrimiento a una velocidad de flujo de aproximadamente 5,0 g/min. Operación del sistema de laqueado, con una velocidad de rotación del bombo de aproximadamente de 19 rpm;

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c) mantener las condiciones de 37,5°C en los núcleos ajustando la temperatura en el termostato; y

d) una vez terminada la solución para laquear apagar la bomba y colocar la temperatura del termostato de aire a 25°C, mantener el bombo girando durante 30 min a 5 rmp para enfriar los núcleos.

11. El uso de la composición obtenida de conformidad con las reivindicaciones 1 a 7 en el tratamiento de la obesidad.

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RESUMEN

La presente invención describe una composición farmacéutica sólida de liberación modificada, que comprende: un compuesto sólido farmacéuticamente activo derivado de una amina simpaticomimetica, particularmente **Clobenzorex** o una de sus sales farmacéuticamente aceptables y un o más agentes diluyente, modificador de liberación, antiadherente, lubricante y de recubrimiento.

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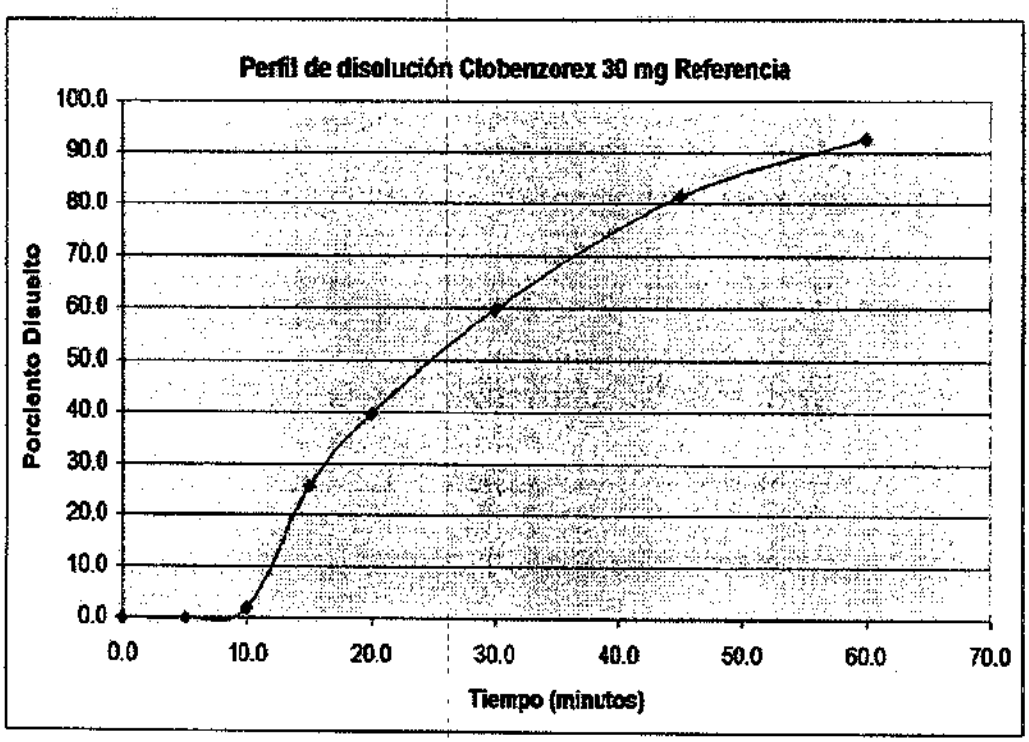

Fig. 1

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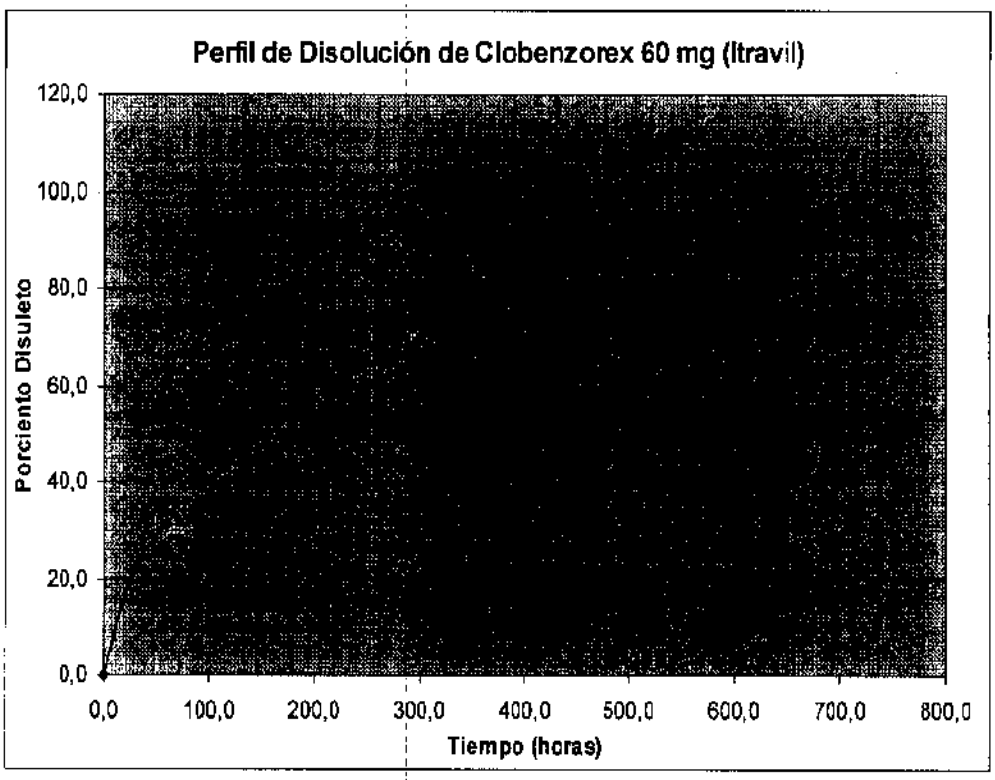

Fig. 2